Engineering Pseudomonas aeruginosa strains towards the production of novel and effective rhamnolipids for use as biocontrol agents

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Universidade do Minho
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Professor Doutor José António Couto Teixeira

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Ana Rodrigues

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.
I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

SUMÁRIO

Manipulação genética da estirpe *Pseudomonas aeruginosa* para uma produção mais eficiente de ramnolípidos como agentes biológicos

A necessidade de substituir os fungicidas químicos por alternativas mais amigas do ambiente, como por exemplo os ramnolípidos, tem vindo a aumentar nos últimos anos. O desenvolvimento de um processo biotecnológico de baixo custo para a sua produção tem sido um desafio. Por este motivo, o principal objetivo desta tese foi desenvolver uma estirpe de *Pseudomonas aeruginosa* para a produção mais eficiente de ramnolípidos como agentes biológicos.

Para desenvolver um meio de cultura mais adequado e barato, foram testados três diferentes resíduos agrícolas para otimizar a produção de ramnolípidos pela estirpe *P. aeruginosa*. O meio de cultura com licor de maceração de milho (10%, v/v), melaço (10%, w/v) e águas russas (25%, v/v) mostrou-se o melhor meio de cultura com uma produção de 4.5 e 5.1 g/L em matraz e reator, respetivamente.

A atividade antifúngica e anti-micotoxigénico dos ramnolípidos foram avaliadas contra diferentes espécies de *Aspergillus*. Os ramnolípidos demonstraram uma elevada atividade antifúngica contra as estirpes *Aspergillus niger* e *Aspergillus carbonarius*. Em relação à estirpe *Aspergillus flavus*, os ramnolípidos apresentaram a capacidade de destruir o conteúdo intracelular das hifas bem como de inibir a produção de aflatoxinas influenciando a expressão dos genes envolvidos na sua síntese. Além disso, foi demonstrado que a atividade antifúngica se deve ao congênere di-ramnolípido e que a atividade dos ramnolípidos purificados se encontra diretamente relacionada com o seu comportamento de agregação.

De acordo com os resultados anteriores, a estirpe *P. aeruginosa* foi geneticamente manipulada com diferentes plasmídeos recombinantes contendo o gene *rhl*C com o intuito de aumentar a produção de di-ramnolípidos, tendo-se obtido resultados muito promissores.

Em suma, os resultados obtidos nesta tese revelaram-se muito interessantes tendo em conta o potencial uso de ramnolípidos como agentes antifúngicos e anti-micotoxigénico, fornecendo ainda informações importantes para o desenvolvimento de uma produção mais eficiente e adequada de ramnolípidos à escala industrial.

Palavras-chaves: Agente antifúngico, *Aspergillus* sps, *Pseudomonas aeruginosa*, Resíduos agrícolas, Ramnolípidos

ABSTRACT

Engineering *Pseudomonas aeruginosa* strains towards the production of novel and effective rhamnolipids for use as biocontrol agents

The demand for "green" compounds, such as rhamnolipids, to replace the chemical fungicides has been growing over the years due to the increasing concern with environmental problems. The development of an efficient bioprocess to produce rhamnolipids, as well as to reduce their production costs has been a challenge. Motivated by this need, the main purpose of this thesis was to engineering *Pseudomonas aeruginosa* towards the production of a novel and effective rhamnolipids production for use as biocontrol agent.

In order to develop an inexpensive and suitable culture medium, the rhamnolipids production by *P. aeruginosa* was performed using three different agro-residues. The most promising results were found using corn steep liquor (CSL) (10%, v/v), molasses (M) (10% w/v) and olive oil mill wastewater (OMW) (25%, w/v). Using this medium, *P. aeruginosa* produced 4.5 and 5.1 g/L of rhamnolipids per liter in flasks and reactor, respectively.

The rhamnolipids produced by *P. aeruginosa* were then evaluated against different *Aspergillus* species to evaluate their antifungal and anti-mycotoxigenic activity. The rhamnolipids showed a high antifungal activity against the strains *Aspergillus niger* and *Aspergillus carbonarius*. Moreover, they were able to damage the hyphae internal content and inhibit the expression of some genes involved in the aflatoxins biosynthesis from *Aspergillus flavus*, thus resulting in a complete inhibition of the aflatoxin production. Furthermore, it was demonstrated that the di-rhamnolipids congeners were responsible for the antifungal activity and that the antifungal activity of the purified rhamnolipids is directly related with their aggregation behavior.

According to the previous results, *P. aeruginosa* strain was engineered with the recombinant plasmids containing the *rhl*C gene to enhance the di-rhamnolipid production and the results obtained were very promising.

Overall, the results gathered in this thesis were found to be very interesting based on the rhamnolipids potential as antifungal and anti-mycotoxigenic agents, providing important insights for the development of a suitable and more efficient rhamnolipids production at an industrial scale.

Keywords: Agro-residues, Antifungal agent, *Aspergillus* sps, *Pseudomonas aeruginosa*, Rhamnolipids

SCIENTIFIC OUTPUTS

Papers:

Gudiña, E. J.*, **Rodrigues, Ana I.***, Freitas, Victor de, A., Z., Teixeira, J. A., Rodrigues, L. R. (2016). Valorization of agro-industrial wastes towards the production of rhamnolipids. Bioresource Technology, 212, 144-150 (doi:10.1016/j.biortech.2016.04.027)

**These authors contributed equally to this work.*

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TABLE OF CONTENTS

GENERAL INTRODUCTION......22

CONTEXT AND MOTIVATION......22

RESEARCH AIMS......24

OUTLINE OF THE THESIS......24

REFERENCES......26

CHAPTER 1......30

RHAMNOLIPIDS: A GREENER ALTERNATIVE TO CHEMICAL SURFACTANTS…......30

CHAPTER 2......56

VALORIZATION OF AGRO-INDUSTRIAL WASTES TOWARDS THE PRODUCTION OF RHAMNOLIPIDS…......56

CHAPTER 3......81

SODIUM CHLORIDE EFFECT ON THE AGGREGATION BEHAVIOUR OF RHAMNOLIPIDS AND THEIR ANTIFUNGAL ACTIVITY......81

CHAPTER 4......104

RHAMNOLIPIDS PRODUCED BY *PSEUDOMONAS AERUGINOSA* AND *BURKHOLDERIA THAILANDENSIS* INHIBIT AFLATOXINS PRODUCTION BY *ASPERGILLUS FLAVUS*......104

CHAPTER 5......124

EFFECT OF RHAMNOLIPIDS IN ASEXUAL DEVELOPMENT AND AFLTOXINS BIOSYNTHESIS......124

CHAPTER 6......145

ENGINEERING *PSEUDOMONAS AERUGINOSA* STRAINS TOWARDS THE PRODUCTION OF DI-RHAMNOLIPIDS 145

CHAPTER 7 170

GENERAL CONCLUSIONS AND FUTURE PRESPECTIVES 170

7.1. GENERAL CONCLUSIONS 171

7.2. FUTURE PRESCPECTIVES 173

ABBREVIATIONS

A

Aflatoxin B_1 (AFB_1)

Aflatoxin B_2 (AFB_2)

Aflatoxin G_1 (AFG_1)

Aflatoxin G_2 (AFG_2)

B

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)

C

Companhia Portuguesa de Amidos, S.A. (COPAM)

Compound Annual Growth Rate (CAGR)

Corn Steep Liquor (CSL)

Corn Steep Liquor and Molasses medium (CSLM)

Critical micelle concentrations (*cmc*)

D

Deoxyribonucleic acid (DNA)

Dihydro-O-methylsterigmatocystin (DHOMST)

Dimethyl sulfoxide (DMSO)

Dynamic Light Scattering (DLS)

E

Electrospray ionization (ESI)

Emulsification index (E_{24})

European Food Safety Authority (EFSA)

European Patent Office (EPO)

F

Fold difference (FD)

Food and Drug Administration (FDA)

G

Generally Recognized as Safe (GRAS)

Gentamycin (Gm)

H

3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs)

Half maximal effective concentration (EC_{50})

High Performance Liquid Chromatography (HPLC)

I

Injection analysis (FIA)

International Agency for Research on Cancer (IARC)

isopropyl-β-D-thiogalactopyranoside (IPTG)

L

Lipopolysaccharides (LPS)

Luria Bertani medium (LB)

M

Malt Extract Agar (MEA)

Mannosylerythritol lipids (MEL)

Mass spectrometry (MS)

Micoteca da Universidade do Minho (MUM)

Microbial Enhanced Oil Recovery (MEOR)

Mineral Salt Medium (MSM)

N

Nutrient Broth (NB)

O

Olive oil mill wastewater (OMW)

O-methylsterigmatocystin (OMST)

Open reading frames (ORFs)

P

Part per million (ppm)

Polydispersity indexes (PDI)

Polymerase Chain Reaction (PCR)

Primer melting temperature (Tm)

Primers efficiency (E)

Q

Quorum Sensing (QS)

R

Refinarias de Açúcar Reunidas (RAR)

Relative standard deviation (RSD)

Retention factor (Rf)

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Rhamnolipid (RL)

Ribonucleic acid (RNA)

S

Sodium chloride (NaCl)

Surface tension (ST)

T

Tetracycline (Tc)

Thin Layer Chromatography (TLC)

Threshold cycle (Ct)

Trace Element Solution (TES)

Transmission Electron Microscopy (TEM)

U

United States Dollar (USD)

United States of America (USA)

LIST OF FIGURES

Chapter 1

Figure 1.1. Structural classification of microbial biosurfactants. **34**

Figure 1.2. General structures of mono- and di-rhamnolipids, m, n:8-24. ....**39**

Figure 1.3. The rhamnolipids metabolic network adapted from Soberón-Chávez et al. (2005) and Chong and Li (2017). **43**

Chapter 2

Figure 2.1. Evolution of the surface tension (mN/m), emulsifying activity (E_{24}, %) and biomass concentration (g dry weight/L) in fermentation preformed with *Pseudomonas aeruginosa* #112 grown in reactor with the culture medium CSLM. The continuous line represents the surface tension of the cell-free supernatants without dilution and the dotted line in surface tension of cell-free supernatants diluted 100 times with demineralized water. Results represent the average of three independent experiments ± standard deviation.....**70**

Figure 2.2. Evolution of the surface tension (mN/m), emulsifying activity (E_{24}, %) and biomass concentration (g dry weight/L) in fermentation preformed with *Pseudomonas aeruginosa* #112 grown in reactor with the culture medium CSLM supplemented with 25% of OMW. The continuous line represents the surface tension of the cell-free supernatants without dilution and the dotted line in surface tension of cell-free supernatants diluted 100 times with demineralized water. Results represent the average of three independent experiments ± standard deviation. **70**

Chapter 3

Figure 3.1 Confocal scanning laser microscopy images showing the self-assembled structures formed by crude rhamnolipid mixture (A, B), mono-rhamnolipids (C) and di-rhamnolipids (D) in the presence of 875 mM NaCl. Nile red was used as fluorescence probe. **97**

Chapter 4

Figure 4.1. Antifungal activity of the crude rhamnolipid mixture produced by *Pseudomonas aeruginosa* #112 at different concentrations against *Aspergillus flavus* MUM 17.14. A: 0 mg_{RL}/L; B: 3000 mg_{RL}/L; C: 1500 mg_{RL}/L; D: 750 mg_{RL}/L; E: 375 mg_{RL}/L. **113**

Chapter 5

Figure 5.1. Transmission electron microscopy (TEM) sections of *Aspergillus flavus* MUM 17.14 non-treated and treated with 1.5 g/L of rhamnolipids produced by *Pseudomonas aeruginosa* #112, after 5 days at 25ºC: (A, B) non-treated conidiophore and hyphae, (C, D) conidiophore and hyphae treated with 1.5 g/L of rhamnolipids. CW: cell-wall, CM: cell membrane, Gc: Golgi´s complex, M: mitochondria, L: lipid content. **135**

Figure 5.2. Expression of the genes (*brl*A, *aba*A and *wet*A) involved in aflatoxin biosynthesis in *Aspergillus flavus* treated with 1.5 g/L of rhamnolipids produced by *Pseudomonas aeruginosa* #112. The results represent four independent experiments with three replicates each. The *A. flavus* β-tubulin gene was used as an internal control to normalize the expression data. NS denotes no significant differences at $p > 0.05$ and * significant differences at $p < 0.05$ as determined by Student´s t-test. **137**

Figure 5.3. Expression of the genes (*afl*R, *afl*S, *afl*C and *afl*Q) involved in aflatoxin biosynthesis in *Aspergillus flavus* treated with 1.5 g/L of rhamnolipids produced by *Pseudomonas aeruginosa* #112. The results represent four independent experiments with three replicates each. The *A. flavus* β-tubulin gene was used as an internal control to normalize the expression data. NS denotes no significant differences at $p > 0.05$, * significant differences at $p < 0.05$ and *** significant differences at $p < 0.001$ as determined by Student´s t-test. **139**

Chapter 6

Figure 6.1. TLC of the mono- (**M**) and di-rhamnolipids (**D**) profile at the end of the fermentation at 37ºC at 180 rpm by *P. aeruginosa* #2 (**WT**) and the transformants of *P. aeruginosa* with pVLT31_PsRhlC with different concentrations of IPTG (0.0, 0.1, 0.2, 0.4, 0.8 and 1.0 mM of IPTG).. **154**

Figure 6.S1. TLC of the mono- (**M**) and di-rhamnolipids (**D**) profile at the end of the fermentation at 37ºC at 180 rpm by *P. aeruginosa* #2 (**WT**) and two transformants of *P. aeruginosa* with pVLT31_BRhlC with 0.4 mM of IPTG (**2** and **3**). **169**

LIST OF TABLES

Chapter 1

Table 1.1. Biosurfactant production using different low-cost substrates. **37**

Chapter 2

Table 2.1. Characterization of OMW used in this work. Results represent the average ± standard deviation of three independent measurements. **64**

Table 2.2. Surface Tension values (ST, mN/m), emulsifying indexes (E_{24}, %), rhamnolipid production ([RL], g/L) and critical micelle concentrations (*cmc*, mg/L) obtained with *Pseudomonas aeruginosa* #112 grown in CLSM medium and CSLM supplemented with different concentrations of OMW. The concentration of total carbohydrates and protein in the different culture media is also given. Results represent the average of three independent experiments ± standard deviation. **67**

Table 2.3. Surface tension (mN/m), emulsifying indexes (E_{24}, %), rhamnolipids production ([RL], g/L) and critical micelle concentrations (*cmc*, mg/L) obtained with *Pseudomonas aeruginosa* #112 grown in CSLM medium and CSLM supplemented with 25 % of OMW in flasks and reactor. Results represent the average of three independent experiments ± standard deviation. **69**

Table 2.4. Rhamnolipid congeners identified as $[M-H]^-$ ions by mass spectrometry in the different rhamnolipid mixtures produced by *Pseudomonas aeruginosa* #112. .... **73**

Table 2.5. Relative abundance (%) of rhamnolipid congeners produced by *Pseudomonas aeruginosa* #112 with the different culture media, in fermentations performed in flasks and reactor. **74**

Chapter 3

Table 3.1. Growth inhibition percentages obtained for *Aspergillus niger* MUM 92.13 with crude rhamnolipid mixture produce by *Pseudomonas aeruginosa* #112. The assays preformed at different NaCl concentrations, at 25ºC for 5 days. The results represent the average of the three independent experiments ± standard deviation. [RL]: concentration of crude rhamnolipid mixture. NT: not tested. **90**

Table 3.2. Growth inhibition percentages obtained for *Aspergillus carbonarius* MUM 05.18 with crude rhamnolipid mixture produce by *Pseudomonas aeruginosa* #112. The assays preformed at different NaCl concentrations, at 25ºC for 5 days. The results represent the average of the three independent experiments ± standard deviation. [RL]: concentration of crude rhamnolipid mixture. NT: not tested. **91**

Table 3.3. Growth inhibition percentages obtained for *Aspergillus niger* MUM 92.13 with the purified mono-rhamnolipid and di-rhamnolipid congeners. The assays were performed at different of purified rhamnolipid congeners with and without NaCl, at 25ºC for 5 days. The results represent the average of three independent experiments ± standard deviation. [RL]: concentration of mono-rhamnolipid or di-rhamnolipid congeners. NT: not tested. **93**

Table 3.4. Growth inhibition percentages obtained for *Aspergillus carbonarius* MUM 05.18 with the purified mono-rhamnolipid and di-rhamnolipid congeners. The assays were performed at different of purified rhamnolipid congeners with and without NaCl, at 25ºC for 5 days. The results represent the average of three independent experiments ± standard deviation. [RL]: concentration of mono-rhamnolipid or di-rhamnolipid congeners. NT: not tested. **93**

Table 3.5. Effect of NaCl on the micellar size distribution of the crude rhamnolipid mixture and the mono-rhamnolipid and di-rhamnolipid congeners determined by DLS analysis. The concentrations of the crude rhamnolipid mixture, mono-rhamnolipids and di-rhamnolipids were 1.5, 1.0 and 0.5 g/L, respectively. The results represent the average of 10 measurements ± standard deviation. ND: not determined. **96**

Chapter 4

Table 4.1. Growth inhibition percentages obtained for *Aspergillus flavus* MUM 17.14 with the crude rhamnolipid mixtures produced by *Burkholderia thailandensis* E264 (RL_{Bt}) and *Pseudomonas aeruginosa* #112 (RL_{Pa}), and with the purified mono- and di-rhamnolipids (from *P. aeruginosa* #112) at different concentrations. The assays were performed at 25°C for 5 days. The results represent the average of three independent experiments ± standard deviation. **112**

Table 4.2. Aflatoxin B_1 (AFB_1) concentration and percentage of AFB_1 reduction (relative to the control) in cultures of *Aspergillus flavus* MUM 17.14 grown in the presence of crude rhamnolipid mixtures produced by *Burkholderia thailandensis* E264 (RL_{Bt}) and *Pseudomonas aeruginosa* #112 (RL_{Pa}), and in the presence of purified mono- and di-rhamnolipids (from *P. aeruginosa* #112) at different concentrations. The results represent the average of three independent experiments ± standard deviation. **116**

Chapter 5

Table 5.1. Gene-specific primers from aflatoxins biosynthesis and sporulation used for RT-PCR. [*E*]: primers efficiency. [Tm]: primer melting temperature. **130**

Chapter 6

Table 6.1. PCR primers, primer melting temperature (Tm), restriction enzymes and PCR conditions used to amplify the *rhl*C gene, and length of products obtained for the strains *Pseudomonas aeruginosa* #112 and *Burkholderia thailandensis* E264 .... **151**

Table 6.2. Surface Tension values (ST, mN/m), Surface Tension diluted 10 times with demineralized water (ST^{-1}, mN/m), rhamnolipid production ([RL], g/L) and critical micelle concentrations (*cmc*, mg/L) obtained with *Pseudomonas aeruginosa* #2 and the different transformants grown in LB medium at the optimal fermentation time. Results represent the average of three independent experiments ± standard deviation. **156**

Table 6.3. Relative abundance (%) of rhamnolipids produced by *Pseudomonas aeruginosa* #2 (WT) and the different transformants, obtained at the end of the fermentation performed in flasks, identified as $[M\text{-}H]^-$ ions by mass spectrometry..**159**

GENERAL INTRODUCTION

Context and motivation

Surfactants are part of the most versatile group of chemicals used in numerous industries, to decrease the surface or interfacial tension between two phases with different polarities, in the form of emulsifiers, detergents, wetting agents, dispersants, or foaming agents (Geys et al., 2014; Gudiña et al., 2015; Mulligan, 2005). The global surfactants market demand was 15 million tons in 2014 and it is expected to growth at Compound Annual Growth Rate (CAGR) of 4.3% from 2015 to 2022 (Grand View Research, 2015). Most of the surfactants commercially available are derived from petrochemical sources, which make them one of the biggest environmentally polluting compounds (Vaz et al., 2012). Inevitably, the surfactants used in different household activity and industries, such as agriculture, are discarded to wastewater-treatment or discarded directly to water ending up to be dispersed into different environment elements (Olkowska et al., 2014). On the other hand, the fluctuating crude oil prices and the rising of environmental concerns are expected to hinder the surfactant market.

In this sense, the increasing of consumer awareness towards the use of eco-friendly products is driving the demand for biosurfactants, as well as the various regulations imposed by many economies like European Union that has instigated the industries to invest in environmentally-friendly bio-based technologies (Grand View Research, 2015). Consequently, the global biosurfactant market is expected to reach 7950 million USD by 2026 growing at a CAGR of 6.9% (Research and Markets, 2019). Biosurfactants are amphiphilic compounds produced by different microorganisms (Joshi et al., 2008; Luna et al., 2013; Pereira et al., 2013). Their environmentally-friendly properties such as high biodegradability and low toxicity make biosurfactants a good “green” alternative to the chemical surfactants (Bharali et al., 2013; Gudiña et al., 2015; Nalini and Parthasarathi, 2014). One of the advantageous potential applications of biosurfactants is in the agriculture industry, in which they could have a promising role by replacing the pollutant surfactants applied in the million-dollar pesticide industry. In agriculture, biosurfactants can contribute for the improvement of soil quality, plant pathogen elimination and beneficial plant microbe interaction (Sachdev and Cameotra, 2013). Biosurfactants are a structurally

diverse group of molecules produced by microorganisms, which can use a wide range of organic compounds as carbon and nitrogen sources.

Rhamnolipids are the among the most effective biosurfactants which find applications in different sectors, including the pharmaceutical, agriculture or petroleum industries, among others (Gudiña et al., 2015; Henkel et al., 2012). Due to their properties, they can be used as wetting, sticker or dispersal agents for application in fungicides or pesticides. Their fungicide and bactericide activities make them useful against certain agricultural pests and to protect foods from spoilage during storage (Kim et al., 2000; Nalini and Parthasarathi, 2014; Sha et al., 2012). In fact, it is estimated that 5 to 10% of the World food's production is lost due to fungal contamination and increasing the agricultural productivity to meet ever growing food demands of human population is a matter of great concern for all countries. Despite of the economic losses contamination by fungi can be harmful for human and animal health due to the presence of mycotoxins produced by different fungi (Bennett and Klich, 2003; Mnif et al., 2015). Mycotoxins are carcinogenic compounds and for that reason there is a concern in reducing their concentration in food and feed to below the levels imposed by the regulatory authorities of which country (EFSA, 2009). Nevertheless, the demand for novel, environmentally-friendly and more effective antifungal and anti-mycotoxigenic agents is urgent due to the development of fungal resistance strains and several environmental effects of long-time applications of chemical surfactants. Despite the rhamnolipid potential their effect on mycotoxin production has never been evaluated.

Regardless of the increasing of the interest in rhamnolipids, the high costs associated to their production is a hindrance to their industrial scale production. It is estimated that the culture media represents 30 to 50% of the total biosurfactant production costs. One of the strategies adopted to reduce the price of culture media could be the formulation of a culture medium using agro-industrial wastes which are the most widely available renewable resource on earth (Chapla et al., 2012; Nurfarahin et al., 2018). Adding value to agro-industrial wastes can provide the reduction of negative emissions, discharge or disposal impact, besides solving an environmental problem and generating additional profits (Beltrán-Ramírez et al., 2019). Subsequently, the understanding of the rhamnolipids biosynthesis may help in the development of "novel strains" able to produce at high yields, reduce the formation of by-products and induce a specific rhamnolipids production (Lovaglio et al., 2015; Müller et al., 2012).

Research aims

The main aims of this thesis were:

1. to produce, recover and separate the different rhamnolipids (mono- and di-rhamnolipids) produced by *Pseudomonas aeruginosa* #112 using a culture media formulated with agro-industrial wastes (e. g. corn steep liquor (CSL), molasses and olive oil mill wastewater (OMW)).

2. to evaluate the antifungal activity of the rhamnolipids (crude mixture and individual congeners) produced by *P. aeruginosa* #112 against different fungi reported as food contaminants and mycotoxigenic.

3. to evaluate the effect of the rhamnolipids (crude mixture and individual homologues) produced by *P. aeruginosa* #112 and *Burkholderia thailandensis* E264 in the aflatoxin production by *Aspergillus flavus* MUM 17.14.

4. to study the effect of the rhamnolipids produced by *P. aeruginosa* #112 on the expression of the genes involved in the aflatoxin biosynthesis and asexual development in *A. flavus* MUM 17.14.

5. to engineer *P. aeruginosa* strains towards the production of novel and effective rhamnolipids that hold the most interest biological activity according to the previous results.

Outline of the thesis

In addition to the General Introduction, this thesis is organized in 7 chapters that cover the research aims stated above. The research aims are covered as follows:

- in Chapter 1, an overview of the side effects of the extensive use of chemical surfactants in the environment and the necessity of replacing them with the a good environmentally-friendly alternative like biosurfactants is provided, with a special focus on rhamnolipids. The rhamnolipids advantageous properties and different applications are highlighted. Moreover, the main obstacles and recent advances

towards solutions to improve the rhamnolipids production in order to make them economically attractive are discussed.

- in Chapter 2, different agro-residues as culture media components were optimized for the rhamnolipids production by *P. aeruginosa* #112 in flasks and reactor. The rhamnolipids production was evaluated by surface tension and emulsification index values. The chemical composition of the agro-residues was studied. The chemical composition of the rhamnolipids mixture produced using different agro-residues in the culture media were characterized by mass spectrometry (ESI-MS). The quantification of rhamnolipids produced were determined by dry-weight of the freeze-dried after their purification by adsorption chromatography using the polystyrene resin Amberlite XAD-2.

- in Chapter 3, the antifungal activity of the rhamnolipids (cell-free supernatant, crude mixture and congeners) produced by *P. aeruginosa* in the best agro-residues medium was evaluated against *Aspergillus niger* MUM 92.13 and *Aspergillus carbonarius* MUM 05.18. The congeners were separated and purified using a silica gel column chromatography with different mobile phases. The antifungal activity was performed in Petri dishes containing MEA medium with different rhamnolipids and congeners concentration and determined by measuring the diameter of the growth zone. Moreover, the effect of NaCl in the rhamnolipids antifungal activity and aggregation behavior are discussed. The aggregation behavior of the crude rhamnolipids mixture and congeners was analyzed through dynamic light scattering (DLS) and confirmed by confocal scanning laser microscopy.

- in Chapter 4, the effect of the rhamnolipids produced by *P. aeruginosa* #112 and *B. thailandensis* E264 on the growth and aflatoxin production by *A. flavus* MUM 17.14 was evaluated. The antifungal activity was assessed as previous describe. The aflatoxins produced were recovered at the end of the antifungal assay with extraction solution and quantified by high performance liquid chromatography (HPLC).

- in Chapter 5, the effect of the rhamnolipids produced by *P. aeruginosa* in the expression of different genes involved in the aflatoxin biosynthesis and asexual

development by *A. flavus* MUM 17.14 was evaluated. The spores were extracted at the end of the antifungal activity and quantified using a Neubauer improved cell counter. The internal structures of hyphae and conidiophores were analyzed by transmission electron microscopy (TEM).

- in Chapter 6, *P. aeruginosa* was transformed with three different recombinant plasmids in order to increase the di-rhamnolipids production (the congener responsible for the antifungal activity). The best transformants were selected based on the analysis of the surface tension, critical micelle concentrations and production yields. The rhamnolipids were recovered at the end of the fermentation and purified as describe above. The rhamnolipids production profile were evaluated through thin layer chromatography (TLC). The rhamnolipids produced by the different transformants were analyzed and the relative percentage quantified by ESI-MS.

- in Chapter 7, the main conclusions are summarized and future perspectives from the current work are addressed.

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CHAPTER 1

RHAMNOLIPIDS: A GREENER ALTERNATIVE TO CHEMICAL SURFACTANTS

ABSTRACT

The Earth planet is currently facing a huge environmental issue. The environmental problems like water pollution, air pollution, soil and land pollution, global warming and many more affect our daily life. The necessity of finding good "green" alternatives have gained a lot of attention in the past years. The consumers are more awareness of this problem and are searching for these green alternatives for their daily chemical's compounds. Surfactants are chemical compounds, derived from petrochemicals, used in diverse products and different industries due to their proprieties that include detergency power, wetting ability and foaming capacity. A good alternative to replace these compounds could be the use of biosurfactants. Biosurfactants are compounds that can be synthesized by different microorganisms and are very promising and interesting molecules as they can be produced from renewable resources using sustainable processes and are biologically degradable. Rhamnolipids, one type of biosurfactants, are among the most effective biosurfactants that have been reported due to their low minimum surface tension values, high emulsifying activity and low critical micelle concentrations (*cmc*). The main obstacle to a wider use of rhamnolipids is the cost associated to their production which make them economically less attractive than their chemical analogues. Strategies to overcome this issue include using agro-industrial wastes and/or by-products as substrates and also design and construct new microorganisms able to metabolize those substrates and efficiently produce biosurfactants. This review explores the technical and economic viability of biosurfactants production from renewable substrates, specifically focusing on rhamnolipids. Moreover, next-generation rhamnolipid-producing strains engineered to reduce the amounts of by-products, increase metabolic spectrum and regulate rhamnolipids synthesis, will be discussed.

Keywords: Biosurfactants, Rhamnolipids, Biocontrol Agent, Renewable substrates, Metabolic engineering

CONTENTS

1.1. INTRODUCTION....32

1.2. BIOSURFACTANTS....33

1.3. RHAMNOLIPIDS....39

1.3.1. RHAMNOLIPIDS PRODUCTION....40

1.3.2. GENETICS AND BIOSYNTHESIS OF RHAMNOLIPIDS....41

1.3.3. POTENTIAL APPLICATIONS OF RHAMNOLIPIDS....45

1.4. CONCLUSIONS AND PERSPECTIVES....46

1.5. REFERENCES....47

1.1. INTRODUCTION

Surfactants are known since ancient times, and the earliest evidence of soap-making dates back to the Babylonians, 2800 years BC (Geys et al., 2014). Surfactants are an important class of chemical compounds included in many of the everyday products we use, with a total worldwide production over 15 million tons per year (Henkel et al., 2012). In 2012, the global surfactant market generated profits of 27 billion USD (Geys et al., 2014), and in 2017 accounted 44 billion USD, and it is expected to reach 66 billion USD by 2025 registering a CARG of 5.4% from 2018 to 2025 (Allied Market Research, 2019). Surfactants can be found in detergents, laundry formulations, household cleaning products, cosmetics, herbicides, or pesticides, and are also applied in bioremediation, agriculture, food, pharmaceutical, textile, paper and petroleum industries, among others (Geys et al., 2014; Gudiña et al., 2015a). Surfactants are used for these applications based on their properties, namely the ability to reduce the surface and the interfacial tensions, increase solubility of hydrophobic and water-insoluble compounds, detergency power, wetting ability and foaming capacity (Mulligan, 2005). They are amphiphilic molecules that contain hydrophilic and hydrophobic groups, which, by accumulating at the interfaces, reduce the surface or interfacial tension between two phases with different polarities. The most conventional surfactants available nowadays are derived from petrochemical sources, which turn them expensive and hazardous to the environment due to their recalcitrant nature (Vaz et al., 2012). The increasing environmental concerns of consumers led to a resurrection of the industrial interest in bio-based compounds, and notably in surfactants (Henkel et al., 2012; Müller et al., 2012). The development of sustainable bioprocesses to produce 'green' products alternative to those chemicals has significantly increased since the beginning of the new millennium. Among these 'green' alternatives, biosurfactants hold a great promise. These compounds are analogous to their chemical counterparts and are synthesized by different microorganisms. However, the application of biotechnologically produced biosurfactants is restricted to certain areas, since their production process is still not economically competitive. Their high production costs are mainly due to the use of high-priced substrates, the relatively low product yields and the expensive downstream-processing (Henkel et al., 2012).

1.2. BIOSURFACTANTS

In the last years, biosurfactants gained considerable attention, since sustainable production processes became more significant, highlighting their potential to replace synthetic surfactants in many applications. The biosurfactant market was 344 kilo tons in 2013 (Grand View Research, 2014), and according with the Global Biosurfactant Market in 2017 it was accounted 4350 million USD and by 2026 it is expected to reach 7950 million USD growing at a CAGR of 6.9% during the forecast period (Research and Markets, 2019). Asia is the fastest growing market because of the largest textile, cosmetic and agriculture industries but, in Europe, the biosurfactant market is expected to increase due to the rising awareness among the costumers towards the protection of the environment from hazard chemical compounds (Research and Markets, 2019). The scientific community accepts two different ways to refer to biosurfactant, namely (a) the surfactants obtained from renewable resources and, (b) the compounds produced by microorganisms (Henkel et al., 2012). This review will only focus in the biosurfactants produced by microorganisms.

Biosurfactants comprise a structurally diverse group of surface active molecules synthesized by different microorganisms, including bacteria, yeasts and filamentous fungi (Joshi et al., 2008; Pereira et al., 2013). They exhibit complex amphipathic structures, comprising both hydrophilic (head) and hydrophobic (tail) groups (Luna et al., 2013). Biosurfactants have attracted an increased attention owing to their advantageous properties such as low toxicity, high biodegradability, good environmental compatibility, high foaming, high selectivity, specific activity at extreme temperatures, pH and salinities, and the ability to be synthesized from renewable feedstocks (Bharali et al., 2013; Gudiña et al., 2015a; Gudiña et al., 2015b; Nalini and Parthasarathi, 2014). Biosurfactants are usually classified in low-molecular-weight compounds (including glycolipids and lipopeptides), and high-molecular-weight polymers (Figure 1.1).The low-molecular weight biosurfactants are known for the ability to reduce surface and interfacial tensions and the high-molecular weights biosurfactants for stabilizing oil-water emulsions (Nurfarahin et al., 2018).

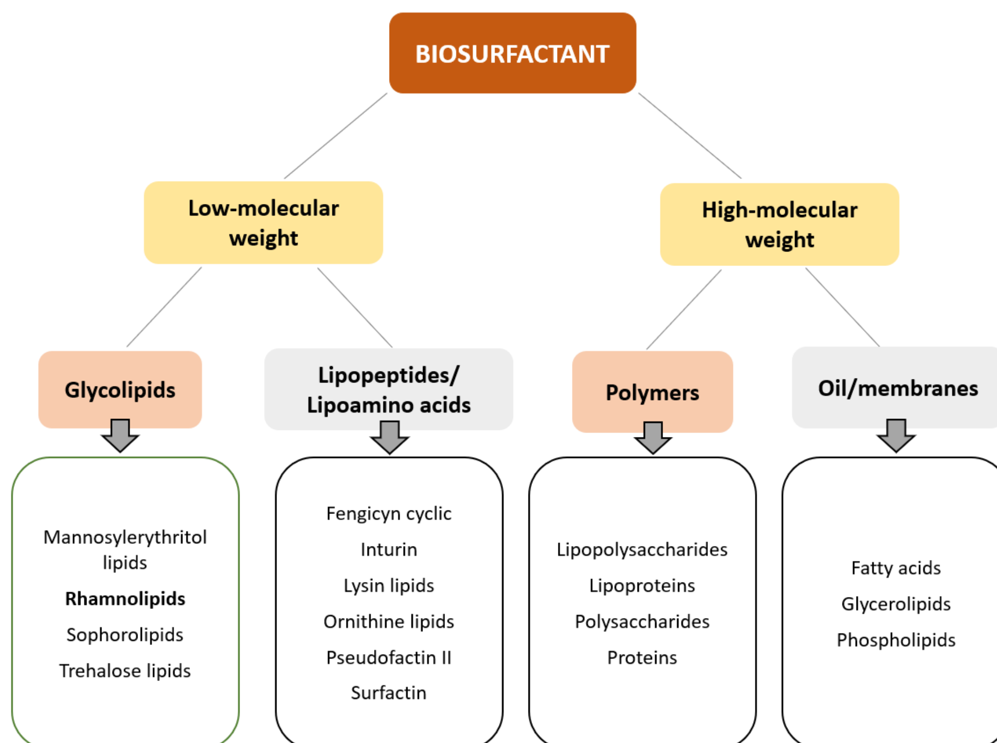

Figure 1.1. Structural classification of microbial biosurfactants.

Despite of this biosurfactants structural diversity, the focus of this review will be on the low-molecular weight glycolipids, particularly on rhamnolipids. Glycolipids have a hydrophilic carbohydrate moiety linked to one or more hydrophobic fatty-acid chains of different lengths. The low-molecular-weight biosurfactants lipopeptides comprise a fatty-acid coupled to a linear or circularized oligo-peptide with varying types and number of amino acids (Jackson et al., 2015; Müller et al., 2012; Ochsner et al., 1994). The best-known glycolipids are mannosylerythritol lipids (MEL), rhamnolipids, sophorolipids and trehalose lipids. The glycolipid MEL is produced by *Pseudozyma* yeasts and their structure contain 4-O-β-D-mannopyranosyl-meso-erythritol and fatty acids (8 to 12 carbons) as the hydrophilic and hydrophobic moieties, respectively (Rodrigues, 2015). Rhamnolipids are a widely studied glycolipid mainly produced by *Pseudomonas sp.* and *Burkholderia sp.*, which consist in one or two rhamnose molecules link to one or two fatty acid chains (Gudiña et al., 2015; Henkel et al., 2012; Pereira et al., 2012). Sophorolipids are produced by several nonpathogenic yeast species, with *Starmerella bombicola* being the most studied. Sophorolipids are extracellular compounds that can comprise acid or lactonic forms, being the last one a result of internal esterification of the carboxylic acid group to a lactone ring (Akiyode et al., 2016; Vandana and Singh, 2018). Trehalose lipids are

characterized by an extremely high structural diversity, constituted by a disaccharide trehalose linked to mycolic acids. They are mainly produced by Gram-positive bacteria such as *Mycobacterium, Norcadia* and *Corynebacteriumi* (Inès and Dhouha, 2015).

Nowadays, as previously mentioned, a limited number of biosurfactants are produced at an industrial scale, mainly due to their high production costs, resulting from the relatively low productivities and the high prices of the culture media used. Biosurfactants are available to the consumer in the form of household detergents (e.g. as those commercialized by Ecover Ltd., Malle, Belgium) or cosmetics products (e.g. as those commercialized by Givaudan Active Beauty, Switzerland). NatSurFact, Evonik and AGAE technologies are companies who commercialize biosurfactants with different degrees of purity that can serve different purposes Despite of the increasing of the interest to these biosurfactants, their cost is not economical attractive being unable to compete economically with their analogue's surfactants. Beside the microorganism's "machinery" is not capable to produce large amount of biosurfactant, the downstream processing could cost approximately between 60% to 80% of the total production costs which make them quite expensive (Banat et al., 2014). Although the industries like pharmaceutical and cosmetics are able to tolerate the low-volume and high production costs, other industries, such as agriculture industry, who need high volume of low-priced surfactants, is not willing to accept the high prices and low production capacity (Helmy et al., 2011). Also, the lack of biosurfactant suppliers may be barred the future progress since the costumers cannot afford the risks associated with few suppliers. In order to increase their competitiveness in the market in comparison with the chemical surfactants, considerable efforts have been made on the development of economical and sustainable production processes (Benincasa and Accorsini, 2008; Gudiña et al., 2015b; Henkel et al., 2012). It is generally accepted that the culture media can account for up to 30-50% of the total biosurfactant production costs. The composition of the culture medium plays a critical role in the production of biosurfactants; in some cases it is necessary the use of hydrophobic carbon sources (alone or in combination with hydrophilic carbon sources) in order to stimulate biosurfactant production; the selection of the nitrogen source is also important, as well as the ratio of carbon:nitrogen source used (Daverey and Pakshirajan, 2010). One of the solutions could pass by formulated an inexpensive and suitable culture medium including different raw materials such as agro-wastes, hydrocarbons or by-products (Nurfarahin et al., 2018). Consequently, a broad spectrum of low-cost substrates have been evaluated for the production of biosurfactants, including

waste glycerol (Kahraman and Erenler, 2012), residues generated during the olive oil production process (olive mill wastewater (OMW)) (Ramírez et al., 2015; Gudiña et al., 2016), soybean oil (Silva et al., 2014), cheese whey (Daverey and Pakshirajan, 2010), cashew apple juice (Oliveira et al., 2013), molasses (Al-Bahry et al., 2013; Gudiña et al., 2015b) and corn steep liquor (CSL) (Gudiña et al., 2015a; Luna et al., 2013) that can be seen in Table 1.1.

The use of agro-industrial wastes as substrates for biosurfactant production, besides making their production less expensive, can contribute, at the same time, to increase their production yields when compared with the synthetic culture media, as well as to alleviate many industrial waste management problems (Gudiña et al., 2015b).

Another problem usually identified in the production of biosurfactants is the transition of the fermentative bioprocess from the laboratorial to the industrial scale. The foam resulting from the emulsifying activity of the biosurfactants due to the vigorous agitation used in the process can decrease the oxygen transfer to the culture medium and greatly affect the microorganism growth. This can be solved by adding, for example, an anti-foaming agent or an hydrocarbon but this will enhance the production costs and can somehow affect the type of molecules being produced (Nurfarahin et al., 2018).

Although biosurfactants exhibit diverse potential industrial applications and display various interesting bioactivities (including cytotoxicity, hemolytic, antimicrobial and anti-adhesive activities) that make them specifically useful for several therapeutic applications, some research is focused on their application in bioemulsification for bioremediation purposes (Jackson et al., 2015). The petroleum industry has been traditionally the main user of surfactants, namely in enhanced oil recovery strategies, due to the effect of surfactants in increasing the solubility of the petroleum components (Mulligan, 2005). Therefore, this industry represents a target user of biosurfactants.

Table 1.1. Biosurfactant production using different low-cost substrates.

Biosurfactant	Culture Medium	Yield (g/L)	References
Rhamnolipids	Glycerol 1 % (w/v) + MSM	1.85	Kahraman and Erenler, 2012
Rhamnolipids	OMW 10% (w/v)	0.191	Ramírez et al., 2015
Rhamnolipids	Molasses 10% (w/v) + CSL 10% (v/v)	3.2	Gudiña et al., 2015b
Rhamnolipids	Molasses 10% (w/v) + CSL 10% (v/v) + OMW 25% (v/v)	4.5	Gudiña et al., 2016
Surfactin	Cashew apple juice + 1.0 g/L $(NH_4)_2SO_4$ + TES*	0.14	Oliveira et al., 2013
Surfactin	Molasses 80 g/L + MSM + TES*	2.29	Al-Bahry et al., 2013
Surfactin	CSL 10% (v/v) + $FeSO_4$ 2mM + $MnSO_4$ 0.2 mM + $MgSO_4$ 0.8 mM	4.8	Gudiña et al., 2015a
Surfactin	OMW 2% (w/v)	0.031	Ramírez et al., 2015
Surfactin + inturin	Potato peels 2% + MSM	3.2	Sharma et al., 2015

*TES - Trace Element Solution
MSM – Mineral Salt Medium

Table 1.1. Biosurfactant production using different low-cost substrates. (*Continuation*)

Biosurfactant	**Culture Medium**	**Yield (g/L)**	**References**
Sophorolipids	Whey 90 g/L + Glucose 10 g/L + Yeast Extract 2 g/L + oleic acid 100 g/L	23.29	Daverey and Pakshirajan, 2010
Lipopeptide	Orange peels 2% + MSM	1.796	Kumar et al., 2016
Anionic molecule (75% lipids and 25% carbohydrates)	Soybean oil 2% + CSL 2% + MSM	-	Silva et al., 2014
Anionic glycolipid (70% lipids and 15% carbohydrates)	Ground nut oil 9% + CSL 9%	9	Luna et al., 2013

*TES - Trace Element Solution
MSM – Mineral Salt Medium

1.3. RHAMNOLIPIDS

Among the glycolipids, rhamnolipids present the higher increase of CAGR due to their growing applications in different fields (Research and Markets, 2019). Rhamnolipids are the most studied glycolipids according with European Patent Office (EPO) as there are registered, until the current year, 144 rhamnolipids patents, 129 sophorolipids patents and 51 MELs patents (EPO, 2019).

Rhamnolipids were first described in 1946 by Bergström and co-workers, and their chemical structure was revealed by two different research groups, namely Jarvis and Johnson (1949) and Edwards and Hayashi (1965). The hydrophobic part of the rhamnolipid molecule comprises one or two fatty acid tails (which can be saturated or unsatured) with chain lengths between C_8 and C_{24}; the different possible combinations result in more than 60 different rhamnolipid congeners (Figure 1.2). The most common congeners are the mono-rhamnolipid L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rha-C_{10}-C_{10}), and the di-rhamnolipid L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxyldecanoate (Rha-Rha-C_{10}-C_{10}). Rhamnolipids are usually produced as mixtures of different congeners, and the ratio of mono- and di-rhamnolipids depends on the producing strain, the composition of the culture medium and the culture conditions (Gudiña et al., 2015b; Müller et al., 2012; Sha et al., 2012). Rhamnolipids are among the most effective biosurfactants due to their low minimum surface tension values (28-30 mN/m), high emulsifying activity (emulsifying indexes up to 60-70%) and low critical micelle concentrations (*cmc*) (10-200 mg/L) (Gudiña et al., 2015b). Furthermore, the rhamnolipids toxicity according to their EC_{50} values (the concentration of a drug that gives half-maximal response) of 20-77 mg/L is about 12 times lower than the values reported for synthetic surfactants (Henkel et al., 2012).

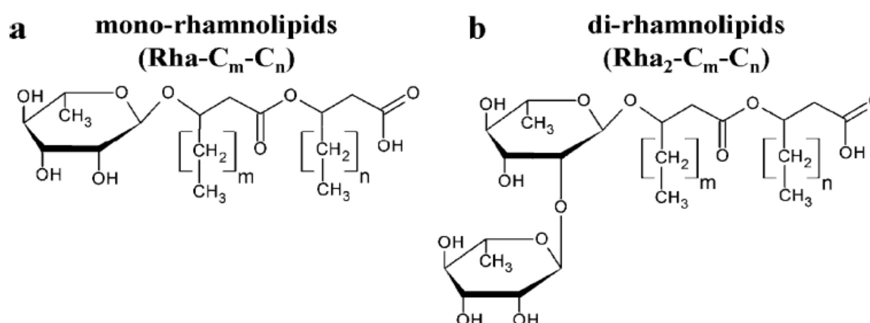

Figure 1.2. General structures of mono- and di-rhamnolipids, m, n:8-24.

1.3.1. RHAMNOLIPIDS PRODUCTION

The increase of ecological concerns with the use of chemical surfactants opened room for rhamnolipids as environmentally-friendly alternatives, although as previously discussed, for their widespread application it will be crucial to reduce their production costs (Kahraman and Erenler, 2012). The costs associated to the downstream processes greatly depend on the final purity degree required. Some industries can use directly the cell-free fermentation broth, leading to a cheaper downstream process, for example in oil recovery, but other industries, such as pharmaceutical industry, need a high level of purity for their applications, thus requiring an expensive downstream process to remove the compounds of interest from the complex fermentation medium (Chong and Li, 2017). Consequently, a doable strategy is to reduce the costs associated with the use of synthetic media by using low-cost substrates. Many researchers have, in some cases, successfully replaced expensive culture media by agro-industrial wastes , such as waste glycerol (Kahraman and Erenler, 2012), wastes from sunflower-oil (Benincasa and Accorsini, 2008), soybean oil (Wang et al., 2007), mango kernel oil (Reddy et al., 2016), molasses (Gudiña et al., 2015b), CSL (Gudiña et al., 2015b) and OMW (Gudiña et al., 2016; Ramírez et al., 2015) for the production of rhamnolipids.

Kahraman and Erenler (2012) used glycerol (1%) as a carbon source to supplement a mineral salts medium ((MSM) for rhamnolipids production. Comparing to the rhamnolipid production using a synthetic medium (Luria-Bertani (LB) medium supplement with glucose (1%)), similar yields under the same agitation conditions (≈7 g/L) were found. Benincasa and Accorsini (2008) used waste from the sunflower-oil process as a component of the culture medium under different conditions. The best result obtained was 7.3 g/L of rhamnolipids using sunflower-oil supplemented with 4 g/L of sodium nitrate. The *cmc* of the product was 120 mg/L. Wang and co-workers (2007) used a MSM supplemented with a TES and soybean oil (2%) as carbon source. The rhamnolipids production was 1.7-1.9 g/L with a *cmc* of 75 mg/L. Reddy and co-workers (2016) produced rhamnolipids using as culture medium mango kernel oil (1%) combined with glucose (1%). The highest rhamnolipid yield obtained was 2.8 g/L with a *cmc* of 80 mg/L. Gudiña and co-workers (2015) evaluated the use of CSL (10%) and molasses (10%) as a culture medium, without the addition of salts or other compounds. In this case, the rhamnolipid production was 3.2 g/L with a *cmc* of 50 mg/L. The same researchers further combined a different waste,

OMW, to the previously optimized culture medium. The best result was achieved using CSL (10%), molasses (10%) and OMW (25%), with a total rhamnolipid production of 4.5 g/L and a *cmc* of 13 mg/L (Gudiña et al., 2016).

Although the use of these wastes revealed to be a good alternative to decrease the production costs of rhamnolipids and contribute to reduce their environmental impact, in most of the cases their single use is not enough, and it is necessary to supplement the culture medium with other compounds to promote the bacterial growth and rhamnolipids production (Table 1.1). Therefore, it is a challenge to find among different wastes the right balance of carbon, nitrogen and lipid contents to develop an optimal culture medium (Helmy et al., 2011). In that sense, the use of CSL, molasses and OMW as the only components of the culture medium represents a meaningful alternative to the use of synthetic media, as no additional compounds are required, and the costs associated to the culture medium are greatly reduced. Moreover, molasses, CSL and OMW besides inexpensive are abundant agro-industrial wastes and by-products (Gudiña et al., 2015b; Nurfarahin et al., 2018). Molasses and CSL are by-products generated during the crystallization of sugar and from corn wet milling industry, respectively. Molasses is a great source of carbon, contains high concentration of carbohydrates (usually about 50%); and CSL is rich in vitamins, minerals, amino acids and proteins, being an important source of nitrogen (Gudiña et al., 2015b; Henkel et al., 2012). OMW is a waste generated during the olive oil extraction that could stimulate the production of rhamnolipids due to their high concentration of long-chain fatty acids (Gudiña et al., 2016).The high nutritional content of these substrates, together with their availability and low price, make them useful products to be used as culture medium or nutrient supplements for microorganisms in diverse industrial fermentation processes.

1.3.2. GENETICS AND BIOSYNTHESIS OF RHAMNOLIPIDS

Rhamnolipids are produced by several bacterial species, including *Pseudomonas* and *Burkholderia* strains, with *Pseudomonas aeruginosa* being the main producer. *P. aeruginosa* strains, despite of the effort to reduce the cost of culture medium, the lower production yields remains an obstacle to their competitiveness within the well-established surfactant market (Geys et al., 2014; Kahraman and Erenler, 2012). Therefore, advanced molecular techniques could be used to bioengineer renewable substrate-consuming bacteria

with the ability of producing inexpensive rhamnolipids with high yields (Lovaglio et al., 2015). Most of the genes involved in the biosynthesis of rhamnolipids, as well as in the regulation of their production, have been identified in *P. aeruginosa* (Figure 1.3) (Ochsner et al., 1994; Pearson et al., 1997; Pesci and Iglewski, 1997). The rhamnose moiety, dTDP-L-rhamnose, is synthesized from glucose-6-phosphate in five consecutive enzymatic steps, catalyzed by the phosphoglucomutae AlgC and Rml enzymes (Nurfarahin et al., 2018; Rahim et al., 2000). Also, these reactions lead to the synthesis of lipopolysaccharides (LPS), the main constituents of the outer cell-wall of Gram-negative bacteria (Rahim et al., 2001; Soberón-Chávez et al., 2005). The lipid moiety precursor, β-hydroxylacyl-ACP, is derived from *de novo* fatty acid synthesis from Acyl-CoA (Zhang et al., 2012). Subsequently, rhamnolipids are synthesized in three enzymatic reactions (by sequential glycosyltransferase reactions) involving two distinct rhamnosyltransferase enzymes. Indeed, three genes involved in the rhamnolipid biosynthesis (*rhlA*, *rhlB* and *rhlC*) have been reported, and their expression is regulated by two quorum sensing (QS) systems. These genes are all transcribed in the same direction, with *rhl*A and *rhl*B being transcribed independently of *rhl*C (Rahim et al., 2000; Rahim et al., 2001). The genes *rhlA* and *rhlB* are responsible for the condensation of one molecule of rhamnose and the fatty acid(s) to form mono-rhamnolipids, and they are organized in a single operon in the *P. aeruginosa* genome. The gene *rhl*A is responsible for the formation of the hydrophobic portion (3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs)) of rhamnolipids, using β- hydroxyacyl-ACP as substrate (Chong and Li, 2017). The membrane-bound rhamnosyltransferase *rhl*B catalyzes the synthesis of mono-rhamnolipids using dTDP-L-rhamnose and the HAAs as precursors (Soberón-Chávez et al., 2005).The gene *rhlC* is responsible for the incorporation of the second rhamnose molecule (to form di-rhamnolipids) and it is located in another region of the chromosome of *P. aeruginosa* (Rahim et al., 2001; Wittgens et al., 2011). The rhamnolipids and their precursors synthesis is dependent of the induction of the related gene products to express the key enzymes necessary for their production (Chong and Li, 2017).

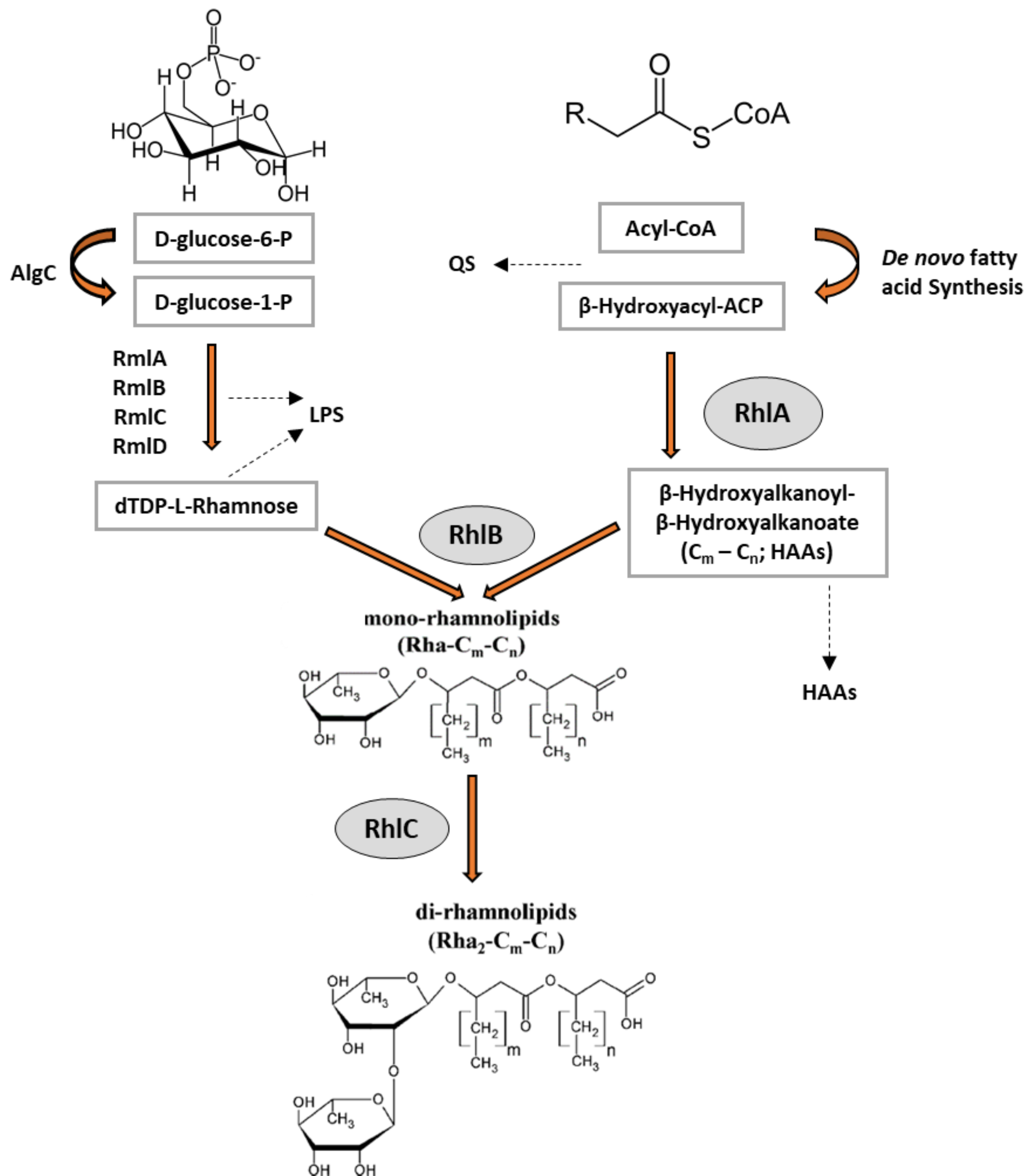

Figure 1.3. The rhamnolipids metabolic network adapted from Soberón-Chávez et al. (2005) and Chong and Li (2017).

In order to avoid the complex regulatory systems existent in *P. aeruginosa*, heterologous expression of the genes involved in the biosynthesis of rhamnolipids has been performed with success in alternative bacterial hosts (such as *Pseudomonas putida*) for rhamnolipid production (Wittgens et al., 2011). Furthermore, some strains of *P. aeruginosa* are opportunistic pathogens capable of causing fatal infections in immunocompromised individuals, such as those with cystic fibrosis, neutropenic cancer and severe burn wounds (Rahim et al., 2001). Recombinant rhamnolipid production generally enables important

advantages from an industrial point of view. Rhamnolipid production in bacteria other than *P. aeruginosa* is an important issue to avoid pathogenicity, complex regulations of rhamnolipids synthesis and to broad the product spectrum (Müller et al., 2012; Wittgens et al., 2011). For that reason, several groups have been working on the development of genetic engineering strategies to allow the production of rhamnolipids in heterologous hosts. Wang and co-workers (2007) reported that heterologous expression of the *rhlAB* genes in the *Escherichia coli* strains BL21 and TnERAB led them to produce rhamnolipids. Ochsner and co-workers (1995) evaluated rhamnolipid production by recombinant *Pseudomonas fluorescens*, *P. putida*, *Pseudomonas oleovorans* and *E. coli*, which were transformed with the *rhlAB* operon. Bahia and co-workers (2018) genetically engineered *Saccharomyces cerevisiae*, a generally recognized as safe (GRAS) yeast, by introducing a complete mono-rhamnolipids pathway from *P. aeruginosa* by using the six enzymes involved (RmlA, RmlB, RmlC, RmlD, *rhl*A and *rhl*B), although in this case the rhamnolipids produced remained inside the cells.

At the same time, several studies reported new bacterial strains with the ability of producing rhamnolipids, including members of the genus *Burkholderia* and thermophilic microorganisms. However, in all these cases, further research will be necessary before they became safe alternatives to *P. aeruginosa* (Abdel-Mawgoud et al., 2010; Guo et al. , 2009; Nott et al., 2013; Toribio et al., 2010). Several *Burkholderia* strains, such as *Burkholderia glumae* and *Burkholderia thailandensis*, have been reported to produce rhamnolipids. *B. thailandensis* is non-pathogenic, whereas *B. glumae* is a plant pathogen. Although *P. aeruginosa* produces a mixture of mono- and di-rhamnolipids, the *Burkholderia* species are known to produce mainly di-rhamnolipids with long fatty acid tails and with relatively low yields (Funston et al., 2017; Mohammed et al., 2018). The use of *B. glumae* presents an advantage, as it is already used at an industrial level for the production of lipases (Nott et al., 2013). Other researchers have been studying the production of rhamnolipids by thermophilic microorganisms such as *Thermus thermophilus*. This species shows the advantage of being non-pathogenic and able to use inexpensive substrates such as sunflower oil. The fact that the cultures are carried out at 75ºC helps to avoid contaminations and contribute to increase the solubility of certain compounds (Pantazaki et al., 2010; Pantazaki et al., 2011).

1.3.3. POTENTIAL APPLICATIONS OF RHAMNOLIPIDS

The potential applications of rhamnolipids include their use in bioremediation, and in the field of oil recovery in the process so-called microbial enhanced oil recovery (MEOR) (Henkel et al., 2012). Rhamnolipids can potentially be used as active compounds in pharmaceuticals (antifungal, antibiotics) or for the anti-adhesive treatment of infusion sets. Rhamnolipids could be used as therapeutic antimicrobial agents. Haba et al. (2014) showed that the surface properties of rhamnolipids contributed to the positive dispersion of the essential oils and thus, increased their availability and antimicrobial activity against *Candida albicans* and *Staphylococcus aureus*. Araujo and co-workers (2016) showed that rhamnolipids exhibit anti-adhesive and anti-biofilm properties. Adhesion of microorganisms was reduced up to 79% on polystyrene, while biofilm formation was reduced up to 83% on stainless steel surfaces treated with rhamnolipids. Another application could be their use as environmentally-friendly pesticides, given their antifungal properties (Kim et al., 2000; Nalini and Parthasarathi, 2014; Sha et al., 2012). Indeed, some rhamnolipids are already being applied in the formulation of a commercial biofungicide (ZONIX™, Jeneil Biosurfactant Company, Saukville, WI, USA) (Henkel et al., 2012).

Contamination by certain molds on food and feed products limit their consumption and are associated to undesirable health effects causing high economic losses despite of the preventive measures adopted to control it (Gerez et al., 2010; Mnif et al., 2015). The extensive use of chemical surfactants to control the molds contamination is endangering the entire environmental balance and is critical to human health (Gerez et al., 2010). The development of environmentally-friendly alternative to the chemical pesticides is one of the main ecological challenges being faced by the microbiologists and plant pathologists. In fact, some biosurfactants have been reported as biocontrol agents, but only few reports describe rhamnolipids for this application. Rhamnolipids have been found to exhibit antagonistic effect against some fungi (Kim et al., 2000; Nalini & Parthasarathi, 2014; Reddy et al., 2016) and demonstrated inhibition of pathogenic fungi resistant to commercial chemical pesticides, being di-rhamnolipids more efficient when compared to mono-rhamnolipids (Sha et al., 2012). Kim and co-workers (2000) showed that the di-rhamnolipid (3- (3-[L-rhamnopyranosyl-(1→2)-α -L-rhamnopyranosyloxy]-decanoyloxy)-decanoic acid) exhibits activity against *Colletotrichum orbiculare*, *Cylindrocarpon destructans, Magnaporthe grisea* and *Phytophthora capsici*. Reddy et al. (2016) studied the antifungal

activity of rhamnolipids against *Phytophthora nicotianae*, *Macrophomina phaseolina* and *Fusarium oxysporium* and they exhibited about 61%, 55% and 64% growth inhibition with corresponding rhamnolipid concentrations of 300, 400 and 450 µg/mL, respectively. Nalini and Parthasarathi (2014) showed that rhamnolipids exhibit antifungal activity against *F. oxysporium* and *Colletotrichum gloeosporioides* at different concentrations (100, 250 and 500 µg/mL). Moreover, rhamnolipids stimulate plant immunity, which can be considered as an alternative strategy to reduce the infection by plant pathogens (Luna et al., 2013).

Another problem associated to the food contamination by molds is the fact that some fungi are known to produce mycotoxins. Species of the genera *Aspergillus*, *Penicillium* and *Fusarium* are among the most important toxigenic fungi and are frequently reported in the literature due to their occurrence in foods and their potential for mycotoxin production (Barrett, 2000; Simas et al., 2007). Mycotoxins are acutely toxic fungal secondary metabolites that can be found in agricultural commodities. Many mycotoxins are carcinogenic, mutagenic, teratogenic, neurotoxic and immunosuppressive (Bennett and Klich, 2003). Therefore, the levels of mycotoxins in food and feed products should be reduced to the lowest technologically possible levels. Accordingly, several physical, chemical and biological methods have been developed to control their concentration (EFSA, 2009). Recently, Veras and co-workers (2016) shown that some *Bacillus* strains can be potential candidates as biocontrol agents for toxigenic fungi due to their capacity to reduce the fungal growth and the levels of mycotoxins produced. Nevertheless, the demand for innovative solutions to reduce the levels of mycotoxins in such products remains an interesting issue.

Considering the increasing amount of pathogens resistant to antibiotics, biosurfactants with antimicrobial activity may become more popular than conventional drugs as an alternative in the near future (Henkel et al., 2012).

1.4. CONCLUSIONS AND PERSPECTIVES

In the last years, a lot of research has been done on the biotechnological production of rhamnolipids and the study of the molecular mechanisms involved in the regulation of their biosynthesis. Rhamnolipids have a good chance to replace surfactants derived from petrochemical resources and can be adopted by the industry as new “green” surfactants. Rhamnolipids are biodegradable, show low toxicity and can be produced from different

wastes, which reduce their production costs and increase their yields. It was demonstrated that waste substrates like sunflower-oil, molasses or CSL can be applied to produce rhamnolipids, as discussed in this review. Also, their antimicrobial and antifungal activities make them interesting for pharmaceutical and agriculture industries. However, there are several obstacles that hinder the large-scale production of rhamnolipids. Next-generation rhamnolipid producing strains could reduce the high costs inherent to rhamnolipids production since they can reduce the formation of by-products, avoid the complex regulation processes involved in their biosynthesis, and increase the substrate and metabolic spectrum. In the future, rhamnolipids may be successful on a commercial large scale and be readily available for the final consumers.

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CHAPTER 2

VALORIZATION OF AGRO-INDUSTRIAL WASTES TOWARDS THE PRODUCTION OF RHAMNOLIPIDS

ABSTRACT

In this chapter, OMW, a residue generated during olive oil extraction, was evaluated as an inducer of rhamnolipid production. Using a medium containing as sole ingredients corn steep liquor (10%, v/v), molasses (10%, w/v) and OMW (25%, v/v), *Pseudomonas aeruginosa* #112 produced 4.5 and 5.1 g of rhamnolipid per liter in flasks and reactor, respectively, with critical micelle concentrations as low as 13 mg/L. Furthermore, in the medium supplemented with OMW, a higher proportion of more hydrophobic rhamnolipid congeners was observed comparing with the same medium without OMW. OMW is a hazardous waste which disposal represents a serious environmental problem; therefore, its valorization as a substrate to produce added-value compounds such as rhamnolipids is of great interest. This is the first report of rhamnolipid production using a mixture of these three agro-industrial residues, which can be useful for the sustainable production of rhamnolipids.

Keywords: Olive oil mill wastewater, Corn Steep Liquor, Molasses, Rhamnolipid, *Pseudomonas aeruginosa*

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CONTENTS

2.1. INTRODUCTION....58

2.2. MATERIAL AND METHODS 60

2.2.1. BACTERIAL STRAIN 60

2.2.2. AGRO-INDUSTRIAL RESIDUES 60

2.2.3. RHAMNOLIPID PRODUCTION OMW WAS EVALUATED AS AN INDUCER OF RHAMNOLIPID PRODUCTION....60

2.2.4. RHAMNOLIPID RECOVERY....61

2.2.5. SURFACE-ACTIVITY DETERMINATION....61

2.2.6. EMULSIFYING ACTIVITY DETERMINATION 62

2.2.7. DETERMINATION OF THE CRITICAL MICELLE CONCENTRATION (*CMC*) .62

2.2.8. RHAMNOLIPID PRODUCTION BY *P. AERUGINOSA* #112 IN REACTOR 62

2.2.9. IDENTIFICATION OF RHAMNOLIPID CONGENERS 63

2.3. RESULTS AND DISCUSSION 63

2.3.1. EFFECT OF OMW ON RHAMNOLIPID PRODUCTION BY *P. AERUGINOSA* #112…… 63

2.3.2. RHAMNOLIPID PRODUCTION BY P. AERUGINOSA #112 IN REACTOR....68

2.3.3. CHARACTERIZATION OF RHAMNOLIPID MIXTURES 71

2.4. CONCLUSIONS 75

2.5. REFERENCES....76

2.1. INTRODUCTION

Surfactants are an important class of amphiphilic compounds. Their hydrophobic and hydrophilic moieties tend to distribute at the interfaces between fluid phases with different polarities, reducing the surface and interfacial tensions. As a result, these compounds facilitate the formation of emulsions, dispersions and foam, making them essential in applications that require emulsification, lubrication, foaming, solubilization of immiscible compounds or phase dispersion. Their uses include the formulation of cleaning products and detergents, applications in food processing, agriculture, environmental remediation or tertiary oil recovery, among others (Mnif and Ghribi, 2015). The global market of surfactants is continuously growing. Their total worldwide production is estimated to be over 15 million tons per year, and it is expected to reach about 24 million tons and 42 120 million USD per year by 2020 (Markets and Markets, 2015). Most of the surfactants available nowadays are derived from petrochemical sources, which besides contributing to environmental pollution due to their low biodegradability, consume nonrenewable resources.

Surface-active compounds synthesized by microorganisms (biosurfactants) display a similar or better performance when compared with chemical surfactants, besides exhibiting higher biodegradability and lower toxicity, being an environmentally-friendly alternative to conventional synthetic surfactants (Pereira et al., 2013; Vaz, et. al., 2012). Among biosurfactants, rhamnolipids have attracted a pronounced attention in the last years. Rhamnolipids are a class of glycolipid biosurfactants produced mainly by *Pseudomonas aeruginosa* strains, which consist of one or two rhamnose molecules linked to one or two β-hydroxy fatty acids (Henkel et al., 2012; Kiran et al., 2016; Lovaglio et al., 2015). Besides their ability to reduce the surface/interfacial tension and their emulsifying activity, rhamnolipids exhibit additional properties when compared with chemical surfactants, including antimicrobial and antitumor activities (Bharali et al. , 2013; Christova et al., 2013), which expands the spectrum of their potential applications, increasing their value. Examples of companies that commercialize rhamnolipids are AGAE Technologies, USA (www.agaetech.com); Rhamnolipid Companies Inc., USA (www.rhamnolipid.com); Jeneil Biotech Inc., USA (www.jeneilbiotech.com); and Urumqi Unite Bio-Technology Co. Ltd., China (www.unite-xj.en.alibaba.com). However, one of the main limitations associated to the commercialization of biosurfactants is their high production costs when compared with

synthetic surfactants. That can be partially overcome by using inexpensive substrates, such as agro-industrial by-products or wastes (Banat et al., 2014; Gudiña et al., 2015; Henkel et al., 2012). Furthermore, if the residues used are environmentally hazardous and their disposal is problematic, their valorization as substrates for biosurfactants production represents an additional advantage. Olive oil is one of the most widely consumed edible oils, being the leading producers Spain, Italy, Greece and Portugal, which account for approximately 98% of its worldwide production (Dermeche et al., 2013; Gonçalves et al., 2009). During its extraction, two different wastes are generated, namely a solid residue and an effluent known as olive mill wastewater (OMW). The main components of OMW, apart from water (83% to 94%), are lipids, carbohydrates, phenolic compounds, organic acids, tannins, pectins and minerals (Dermeche et al., 2013; Meksi et al., 2012). The composition of OMW varies, both qualitatively and quantitatively, according to the olive variety, the climate conditions, the agronomic practices and especially the technology used to extract the oil from the olives, which can be a discontinuous pressing process or a continuous (two- or three-phase) extraction process. In Portugal, the most common is the continuous three-phase extraction process, which uses more water to extract the oil when compared with the two-phase process, and consequently generates higher amounts of OMW (Gonçalves et al., 2009). It is estimated that about 30 million m^3 of OMW are produced each year in the Mediterranean countries (Dermeche et al., 2013; Meksi et al., 2012). The generation of this residue is seasonal, associated to the production of olive oil, which in Portugal usually takes place between December and February. However, in other Mediterranean countries its production can comprise a longer period (from October to March) (Dermeche et al., 2013; Gonçalves et al., 2012). OMW is a significant pollutant and its disposal constitutes an environmental problem, due to its low degradability and its toxicity against microorganisms, plants and aquatic ecosystems (Dermeche et al., 2013).

In this chapter, OMW was evaluated as an inducer of rhamnolipid production by a *P. aeruginosa* strain, both in flasks and reactor. Its inductive effect was evaluated by supplementing a culture medium containing corn steep liquor (CSL) and molasses with OMW at different concentrations. Furthermore, the main rhamnolipid congeners produced were characterized.

2.2. MATERIAL AND METHODS

2.2.1. Bacterial strain

The bacterial strain *P. aeruginosa* #112 was used for rhamnolipid production. This strain, isolated from a crude oil sample obtained from a Brazilian oil field, was previously reported as a promising rhamnolipid producer (Gudiña et al., 2015). It was stored at -80ºC in Luria Bertani (LB) medium supplemented with 20% (v/v) of glycerol. The composition of LB medium was (g/L): NaCl 10.0; tryptone 10.0; yeast extract 5.0; pH 7.0.

2.2.2. Agro-industrial residues

Molasses and CSL were kindly provided by RAR: Refinarias de Açúcar Reunidas, S.A. (Portugal) and COPAM: Companhia Portuguesa de Amidos, S.A. (Portugal), respectively. OMW was obtained from an olive oil mill located in the north of Portugal, which uses a continuous three-phase centrifugation process for olive oil extraction. All the residues were stored at 4ºC until use. The concentration of total carbohydrates and protein in these residues were determined using the phenol-sulfuric and Lowry methods, respectively (Dubois et al., 1956; Lowry et al, 1951). Furthermore, OMW was characterized in terms of pH, total solids, total phenols, total lipids and long-chain fatty acids composition, using methodologies described elsewhere (Amaral et al., 2012; Gonçalves et al., 2009, 2012). Total solids were determined by oven drying (105ºC) to constant weight. Total phenols were evaluated by the Folin–Ciocalteu method and expressed as $mg_{caffeic}$ acid/L. Lipids were extracted with diethyl ether, in a Soxtec^{TM} 8000 extraction unit (FOSS, Hillerød, Denmark). Long-chain fatty acids were determined by gas chromatography.

2.2.3. Rhamnolipid production OMW was evaluated as an inducer of rhamnolipid production

For that purpose, different culture media were prepared containing CSL (10%, v/v) and molasses (10%, w/v), supplemented with OMW at concentrations between 5% and 25% (v/v). All the media were adjusted to pH 7.0. Rhamnolipid production by *P. aeruginosa* #112 was studied in 500 mL flasks containing 200 mL of the different culture media. Each flask was inoculated with 1% (v/v) of a pre-culture of *P. aeruginosa* #112 grown overnight in LB medium, and after that they were incubated at 37ºC and 180 rpm. Samples were taken

at different time points during the fermentation in order to evaluate the production of rhamnolipids. The samples were centrifuged (10 000 x g, 20 min, 20ºC) and the cell-free supernatants were used to measure the surface tension (ST) and to determine the emulsifying activity (E_{24}), as described below. Whenever required, the cell-free supernatants were diluted with demineralized water and the corresponding surface tensions were measured, in order to study the evolution of biosurfactant production. The cultures were maintained until the maximum rhamnolipid production was achieved (according to the surface tension values and the emulsifying indexes). All the experiments were performed in triplicate.

2.2.4. Rhamnolipid recovery

The rhamnolipids produced by *P. aeruginosa* #112 were recovered from the cell-free supernatants obtained at the end of the different fermentations by adsorption chromatography using the polystyrene resin Amberlite XAD-2 (Sigma–Aldrich Co., USA). A 125 ml column was filled with Amberlite XAD-2 and equilibrated with three volumes of 0.1 M potassium phosphate buffer (pH 6.1) at a constant flow rate of 3 ml/min. The cell-free supernatants were adjusted to pH 6.1 and introduced into the column at the same flow rate. Subsequently, the column was washed with three volumes of demineralized water to remove the non-adsorbed compounds. Finally, the biosurfactant adsorbed to the resin was eluted with three volumes of methanol; the methanol was removed using a rotary evaporator at room temperature and the crude biosurfactant was dissolved in a minimal amount of demineralized water and freeze-dried. Rhamnolipid production was determined as dry weight of the freeze-dried purified products and stored at -20ºC for further studies.

2.2.5. Surface-activity determination

The surface tension measurements were performed according to the Ring method as described elsewhere (Harkins and Jordan, 1930) using a KRÜSS K20 Tensiometer (KRÜSS GmbH, Germany) equipped with a 1.9 cm De Noüy platinum ring at room temperature (25ºC).The tensiometer arm is lowered until the ring contacted the surface of the test liquid contained in a respective reservoir and started to raise from the surface of the liquid at a constant speed to a specific height, while the tensiometer registered the force as a function of time and distance. The surface tension is calculated by the maximum force

that represents the detachment of the ring from the surface of the liquid. All the measurements were performed in triplicate.

2.2.6. Emulsifying activity determination

The emulsifying activity was determined by addition of 2 mL of n-hexadecane to the same volume of cell-free culture broth supernatant or rhamnolipids solution in glass test tubes. The tubes were mixed with vortex at high speed for 2 min and incubated at 25ºC for 24h. The emulsion was determined after 24h, and the E_{24} was calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm). All the emulsification indexes were determined in triplicate.

2.2.7. Determination of the critical micelle concentration (*cmc*)

The *cmc* is the concentration of an amphiphilic component in solution at which the formation micelles are initiated. Concentrations ranging from 0.001 to 5 g/L of the freeze-dried rhamnolipids produced by *P. aeruginosa* #112 in the different culture media were prepared with demineralized water and surface tension of each sample was determined by the Ring method at room temperature (20ºC) as describe above. The *cmc* was determined, as described by Gudiña et al. (2012), by plotting the surface tension as a function of the logarithm of biosurfactant concentration and it was found the point intersection between the two lines that best fit through the pre- and post-*cmc* data. All the measurements were performed in triplicate.

2.2.8. Rhamnolipid production by *P. aeruginosa* #112 in reactor

A 5L reactor (BIOSTAT_ A Fermentor, B. Braun Biotech International GmbH, Germany), equipped with agitation, temperature, pO_2 and pH online measurement and control was used. The experiments were performed with 2L working volume at 37ºC. The reactor was inoculated with 1% (v/v) of a pre-culture of *P. aeruginosa* #112 grown overnight in LB medium at 37ºC and 180 rpm. The effect of different agitation (200-450 rpm) and airflow (0.2–1 vvm) rates on rhamnolipid production was studied. Air was injected in the top of the reactor, without contacting the culture medium, to avoid the formation of foam. The fermentations were conducted as batch cultivations without pH control. In order to evaluate bacterial growth and rhamnolipid production, samples were

taken at different time points during the fermentation. The fermentations were maintained until the maximum rhamnolipid production was achieved (according to the ST values and the E_{24}). Surface tension and emulsifying indexes measurements, rhamnolipid recovery and *cmc* calculation were performed as described above. Bacterial growth was determined by measuring the optical density at 600 nm, and biomass concentration (g dry weight/L) was calculated using a calibration curve.

2.2.9. Identification of rhamnolipid congeners

For chemical composition analysis, the freeze-dried biosurfactant samples were prepared in two different ways. First, they were dissolved in methanol, centrifuged (10 000 x g, 5 min), and the final solution was analyzed by direct flow injection. Secondly, the samples were dissolved in water at a concentration of 10 mg/mL and extracted with a chloroform/methanol mixture (2:1, v/v) to achieve a final chloroform/methanol/water ratio of 8:4:3 (v/v/v). The final mixture was centrifuged (10 000 x g, 5 min), and the resulting organic layer analyzed in the same way. The samples were analyzed by electrospray ionization (ESI) mass spectrometry (MS). An aliquot of each sample was injected by flow injection analysis (FIA) into an LTQ-Orbitrap XL mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA). The sample was pumped at 5 µL/min using nitrogen as sheath gas at a flow of 5 (arbitrary unit system). The electrospray conditions were as follows: 3 kV for the spray voltage; 270ºC for the capillary temperature; the tube lens was established at 100 V under optimum analysis conditions. All the organic solvents used were of analytical grade or mass spectrometry grade for Orbitrap analysis, and were purchased from Panreac (Castellar del Vallés, Barcelona, Spain). Milli-Q water was obtained from a water purification system (Millipore, Millerica, MA, USA). Trifluoroacetic acid (HPLC grade) was purchased from Sigma-Aldrich (USA).

2.3. RESULTS AND DISCUSSION

2.3.1. Effect of OMW on rhamnolipid production by *P. aeruginosa* #112

Previously, we developed the culture medium CSL and molasses (CSLM) for rhamnolipid production, containing as sole ingredients the agro-industrial residues CSL (10%, v/v) and molasses (10%, w/v). Using that medium, the isolate *P. aeruginosa* #112

produced 3.2 g of rhamnolipids per liter, with a *cmc* of 50 mg/L (Gudiña et al., 2015). In this work, the agro-industrial waste OMW (which composition is summarized in Table 2.1) was evaluated as an inducer of rhamnolipid production. Although the composition of OMW depends on several factors as previously explained, the results herein showed are in accordance with previous characterizations of OMW obtained from the same region (Amaral et al., 2012; Gonçalves et al., 2009, 2012). As it can be seen in Table 2.1., OMW is a source of long-chain fatty acids, including palmitic (C_{16}), stearic (C_{18}), oleic ($C_{18:1}$) and linoleic acid ($C_{18:2}$), being oleic acid the most abundant (3.3 g/L). It has been reported that using a combination of carbohydrates and long-chain fatty acids, the rhamnolipid production by *P. aeruginosa* strains is higher than using carbohydrates as a single carbon source (Zhang et al., 2012, 2014)

Table 2.1. Characterization of OMW used in this work. Results represent the average ± standard deviation of three independent measurements.

Parameter	**Value**
pH	5.1 ± 0.1
Total solids (g/L)	59.8 ± 3.0
Total phenols (g/L)	2.6 ± 0.1
Total carbohydrates (g/L)	11.7 ± 0.9
Total proteins (g/L)	0.45 ± 0.05
Lipids (g/L)	4.8 ± 0.3
Long-chain fatty acids (mg/L)	
Palmitic acid (C_{16})	513 ± 32
Stearic acid (C_{18})	129 ± 12
Oleic acid ($C_{18:1}$)	3387 ± 89
Linoleic acid ($C_{18:2}$)	284 ± 21

Therefore, OMW can be used as an inexpensive source of long-chain fatty acids to stimulate the production of rhamnolipids. Given the good results obtained previously using the medium CSLM, rhamnolipid production by *P. aeruginosa* #112 was assayed using the

same medium but now supplemented with OMW at different concentrations (5-25%, v/v). The results obtained are shown in Table 2.2. The surface tension of the different media was around 50 mN/m. As can be seen from Table 2.2, a considerable surface tension reduction was observed at the end of the different fermentations. With the medium CSLM without OMW, the maximum rhamnolipid production was achieved after 144 h of growth; however, in all the media supplemented with OMW, the maximum rhamnolipid production was achieved at 168 h. Although the surface tension values obtained at the end of the fermentation with the different media were very similar (around 31 mN/m), as the OMW concentration increased (from 5% to 15%) the amount of rhamnolipid produced also increased, reaching 5.030 ± 0.221 g/L for an OMW concentration of 15%. At the same time, the *cmc* of the rhamnolipids produced decreased, from 50 mg/L up to 34 mg/L. The media containing 20% and 25% of OMW led to the production of lower amounts of rhamnolipids when compared with the media containing 10% and 15% of OMW, although with lower *cmc* values (14-15 mg/L) (Table 2.2). Rhamnolipids are usually produced as mixtures of different compounds (mono- and di-rhamnolipids, containing one or two fatty acids with different chain lengths) (Abdel-Mawgoud et al., 2010). The differences observed in the *cmc* values herein obtained can be due to the different purity of the products recovered, or to differences in the qualitative and/or quantitative composition of the rhamnolipid mixtures produced, as it will be later discussed. In all the cases, the purified rhamnolipids reduced the surface tension of demineralized water from 72 mN/m up to 27 mN/m at concentrations equal or higher than their *cmc*. Regarding the emulsifying activity, the best results were obtained for the culture media supplemented with OMW at concentrations between 10% and 25% (Table 2.2). Several authors reported the use of different agro-industrial wastes or by-products as substrates to produce rhamnolipids by different *Pseudomonas* sp. strains, including orange peels, oil wastes or molasses. Using those substrates, the surface tension of the culture medium was reduced up to 30-33 mN/m, and emulsifying indexes between 59% and 84% were obtained; the amounts of rhamnolipid produced were between 5 and 14 g/L, with *cmc* between 27 and 120 mg/L (Aparna et al., 2012; Benincasa and Accorsini, 2008; George and Jayachandran, 2009; Haba et al., 2003; Lan et al., 2015; Nitschke et al., 2005). However, in most of these cases, the culture medium was supplemented with additional carbon or nitrogen sources, salts or mineral elements, which increases the production costs. Furthermore, in these reports the rhamnolipid yields were calculated indirectly using colorimetric methods such as the orcinol assay, which overestimates between 5 and 9 times the real concentration of rhamnolipids in the samples,

as discussed by Perfumo et al., (2013). Ramírez et al., (2015) reported the production of rhamnolipids by a *P. aeruginosa* strain using 'alperujo' (a solid residue generated during olive oil production using the two-phase extraction process) as the sole carbon source (10%, w/v). However, the culture medium was supplemented with additional nutrients, and the rhamnolipid yield obtained was 0.191 g/L, which is considerably lower when compared with the results herein reported. Supplementing the low-cost culture medium CSLM (which value is about 0.024 €/L) with OMW at different concentrations resulted not only in the production of higher amounts of rhamnolipids, but also in the production of more efficient rhamnolipid mixtures (i.e. with lower *cmc* values).

To the best of our knowledge this is the first time that rhamnolipid production using a combination of CSL, molasses and OMW is reported. Furthermore, these substrates were used without any previous treatment and without additional supplements. Considering the amount of rhamnolipids produced and their *cmc*, it can be concluded that the medium CSLM supplemented with 25% of OMW offered the best results. In *P. aeruginosa*, the expression of the genes *rhl*A and *rhl*B, involved in the biosynthesis of rhamnolipids, is increased up to 600 times when long-chain fatty acids (including oleic, linoleic and stearic acid) are used as co-substrates together with glucose, when compared with the use of glucose as the sole carbon source (Zhang et al., 2012). Furthermore, when carbohydrates are the sole carbon source, the precursors for the synthesis of the lipid moiety of rhamnolipids are provided only by *de novo* fatty acid synthesis. However, in the presence of carbohydrates and long-chain fatty acids, they are supplied by *de novo* fatty acid synthesis and oxidation, which results in a higher production of lipid precursors for rhamnolipid biosynthesis (Zhang et al., 2014). As a result of both inductive processes, rhamnolipid production is increased up to two times when the culture medium contains carbohydrates and long-chain fatty acids (Zhang et al., 2012, 2014). This could explain the inductive effect of OMW on the rhamnolipid production when added to the culture medium CSLM.

Table 2.2. Surface Tension values (ST, mN/m), emulsifying indexes (E_{24}, %), rhamnolipid production ([RL], g/L) and critical micelle concentrations (*cmc*, mg/L) obtained with *Pseudomonas aeruginosa* #112 grown in CLSM medium and CSLM supplemented with different concentrations of OMW. The concentration of total carbohydrates and protein in the different culture media is also given. Results represent the average of three independent experiments ± standard deviation.

Medium	[Carbohydrates] (g/L)	[Protein] (g/L)	Time (h)	ST (mN/m)	E_{24} (%)	[RL] (g/L)	*cmc* (mg/L)
CSLM	56.5	0.56	144	31.4 ± 0.1	59.0 ± 1.5	3.194 ± 0.245	50
CSLM + 5% OMW	57.1	0.58	168	31.3 ± 0.3	58.2 ± 2.5	3.758 ± 0.176	38
CSLM + 10% OMW	57.7	0.60	168	31.4 ± 0.2	63.5 ± 1.4	4.660 ± 0.102	36
CSLM + 15% OMW	58.2	0.63	168	31.3 ± 0.2	64.0 ± 2.1	5.030 ± 0.221	34
CSLM + 20% OMW	58.8	0.65	168	31.0 ± 0.1	63.9 ± 2.0	3.848 ± 0.190	15
CSLM + 25% OMW	59.4	0.67	168	31.0 ± 0.1	64.0 ± 0.5	4.526 ± 0.105	14

2.3.2. Rhamnolipid production by P. aeruginosa #112 in reactor

The media CSLM and CSLM supplemented with 25% of OMW were evaluated for rhamnolipid production in a 5L reactor. Different agitation (200-450 rpm) and aeration (0.2-1 vvm) rates were assayed, and the best results were obtained at 350 rpm and 0.5 vvm, with the air injected in the top of the reactor. With this foam-free production approach, only a thin layer of foam was formed in the upper phase of the medium, similar to that formed in the fermentations performed in flasks. The results obtained are summarized in Table 2.3, Figure 2.1 and Figure 2.2. With both media, the surface tension reductions obtained were higher when compared with the assays performed in flaks; however, the emulsifying indexes were slightly lower. Using the medium CSLM without OMW, the amount of rhamnolipid produced in the reactor was lower when compared with the assays performed in flasks, although it exhibited a lower *cmc* (i.e. it was more efficient).

Regarding the medium CSLM supplemented with 25% of OMW, the amount of rhamnolipid produced in the reactor was higher when compared with the assays performed in flasks, and their *cmc* were similar. Figure 2.1 and Figure 2.2 shows the evolution of cell growth, surface tension and emulsifying activity in these assays. In the case of the medium containing OMW (Figure 2.1), growth and rhamnolipid production started later when compared with the medium CSLM (Figure 2.1), which can be probably due to the toxic effect of some components of OMW (e.g. phenolic compounds).

Table 2.3. Surface tension (mN/m), emulsifying indexes (E_{24}, %), rhamnolipids production ([RL], g/L) and critical micelle concentrations (*cmc*, mg/L) obtained with *Pseudomonas aeruginosa* #112 grown in CSLM medium and CSLM supplemented with 25 % of OMW in flasks and reactor. Results represent the average of three independent experiments ± standard deviation.

Medium		**Time (h)**	**ST (mN/m)**	**E_{24} (%)**	**[RL] (g/L)**	***cmc* (mg/L)**
CSLM	Flask	144	31.4 ± 0.1	59.0 ± 1.5	3.194 ± 0.245	50
	Reactor	96	29.0 ± 0.2	54.2 ± 2.0	2.236 ± 0.103	30
CSLM + 25% OMW	Flask	168	31.0 ± 0.1	64.0 ± 0.5	4.526 ± 0.105	14
	Reactor	168	29.2 ± 0.2	58.4 ± 1.0	5.124 ± 0.125	13

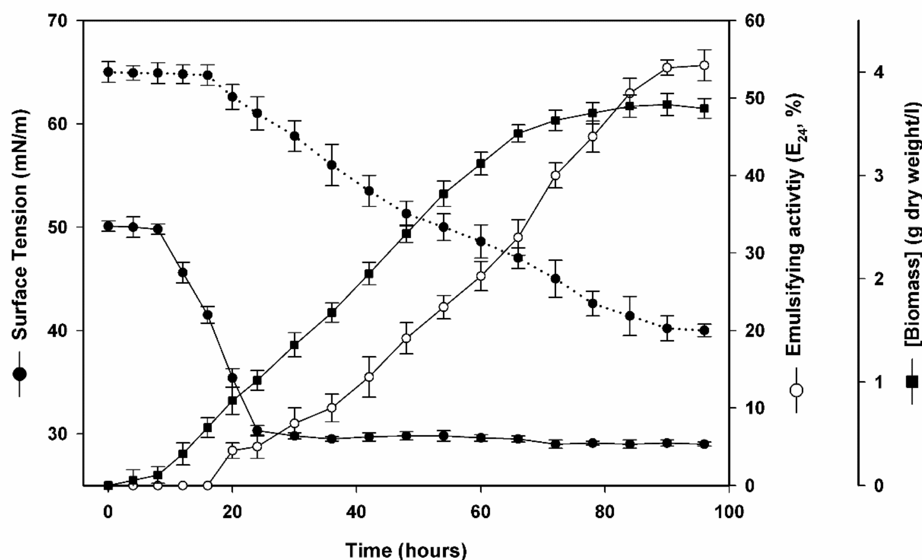

Figure 2.1. Evolution of the surface tension (mN/m), emulsifying activity (E_{24}, %) and biomass concentration (g dry weight/L) in fermentation preformed with *Pseudomonas aeruginosa* #112 grown in reactor with the culture medium CSLM. The continuous line represents the surface tension of the cell-free supernatants without dilution and the dotted line in surface tension of cell-free supernatants diluted 100 times with demineralized water. Results represent the average of three independent experiments ± standard deviation.

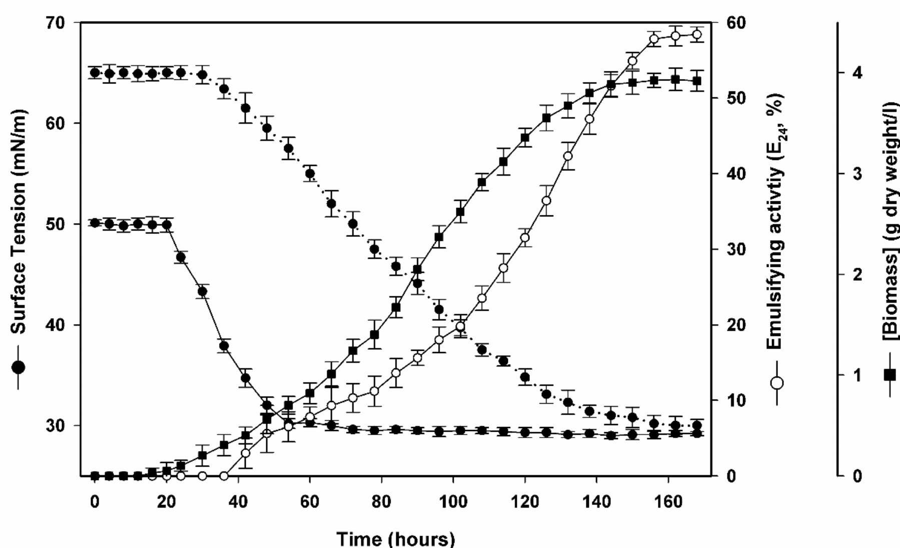

Figure 2.2. Evolution of the surface tension (mN/m), emulsifying activity (E_{24}, %) and biomass concentration (g dry weight/L) in fermentation preformed with *Pseudomonas aeruginosa* #112 grown in reactor with the culture medium CSLM supplemented with 25% of OMW. The continuous line represents the surface tension of the cell-free supernatants without dilution and the dotted line in surface tension of cell-free supernatants diluted 100

times with demineralized water. Results represent the average of three independent experiments ± standard deviation.

With both media, the surface tension of the cell-free supernatants decreased quickly after the lag phase to values around 30 mN/m and then remained almost constant until the end of the fermentation. This means that the amount of rhamnolipids present in the medium was equal or higher than the *cmc*. Consequently, it was necessary to dilute the cell-free supernatants to study the evolution of rhamnolipid production along the fermentations. The surface tension of the 100 times diluted cell-free supernatant continued decreasing until the end of the fermentation in both media, thus indicating a continuous production of rhamnolipids (Figure 2.1 and Figure 2.2). However, the emulsifying indexes measured in the supernatants without dilution continued increasing in both media until the end of the fermentation, meaning that a higher concentration of rhamnolipids is necessary to achieve a high emulsifying activity when compared with that necessary to reduce the surface tension. Regarding the medium CSLM, biomass concentration reached its maximum after 90 h (3.686 ± 0.105 g dry weight/L), while the highest rhamnolipid production (according to the surface tension of 100 times diluted supernatant) was achieved at 96 h. In the case of the medium CSLM supplemented with 25% of OMW, the highest biomass concentration was achieved at 162 h (3.934 ± 0.110 g dry weight/L) and the highest rhamnolipid production at 168 h. In both cases, a parallel relationship was observed between cell growth and rhamnolipid production, which suggests a growth-associated production profile. The same profile was reported for different *P. aeruginosa* strains by other authors (Aparna et al., 2012; Bharal and Konwar, 2011). However, in other cases it has been referred that rhamnolipid production occurs mainly during the stationary growth phase (Haba et al., 2003; Nitschke et al., 2005; Ramírez et al., 2015). Fermentations performed with biosurfactant-producing microorganisms are usually associated to a severe foam production, which can result in the overflow of the culture medium and microbial cells. The use of antifoam agents is not attractive from an economic point of view; furthermore, these compounds are difficult to remove in the downstream processing and can have a negative effect on the biosurfactant production. As an alternative, the foam produced (which contains a high biosurfactant concentration) can be channeled through one of the outputs of the reactor and collected to further extract the biosurfactant, in a process known as foam fractionation. Although this technology has been widely used for biosurfactant production in the laboratory (Sarachat et al., 2010; Willenbacher et al., 2014), it requires a complex design and construction that can affect the scale-up process and its industrial

application. Consequently, the application of a foam-free fermentation strategy as described here can be a better alternative.

2.3.3. Characterization of rhamnolipid mixtures

The rhamnolipid mixtures produced by *P. aeruginosa* #112 in assays performed in flasks and reactor with different media were characterized by mass spectrometry by direct injection in an Orbitrap ESI-MS spectrometer operating in the negative mode. The measured accurate mass of the $[M-H]^-$ pseudomolecular ions was matched with exact mass of the molecular structures expected for mono- and di-rhamnolipids, based on other similar studies (Abdel-Mawgoud et al., 2010; Gudiña et al., 2015), and the mass error was calculated (Table 2.4). According to Table 2.5, in all the cases seven different rhamnolipid congeners were identified, including mono- and di-rhamnolipids.

The most abundant congener was the mono-rhamnolipid Rha-C_{10}-C_{10}, followed by the di-rhamnolipid Rha-Rha-C_{10}-C_{10} and then the mono-rhamnolipid Rha-C_{10}, regardless of the culture media used, both in fermentations performed in flasks or in reactor. These results are in agreement with the results obtained in the positive mode in which the sodium adducts of the rhamnolipids Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10} were also the most abundant ions. The predominant hydroxyl fatty acid was decanoic acid, with trace amounts of octanoic and dodecanoic acids. The main differences observed between the different culture media were in the relative abundance of some rhamnolipid congeners. In assays performed in flasks, supplementing the medium CSLM with OMW resulted in a reduction in the relative abundance of the mono-rhamnolipid Rha-C_{10}; and with the medium supplemented with 15% of OMW, a higher relative abundance of the di-rhamnolipid Rha-Rha-C_{10}-C_{10} was observed when compared with the other two media. Regarding the ratio mono-/di-rhamnolipid, the same value was observed for the media CSLM and CSLM supplemented with 25% of OMW, whereas the medium CSLM supplemented with 15% of OMW exhibited a lower proportion of mono-rhamnolipids.

Table 2.4. Rhamnolipid congeners identified as $[M-H]^-$ ions by mass spectrometry in the different rhamnolipid mixtures produced by *Pseudomonas aeruginosa* #112.

Compound	[M-H]- measured mass	[M-H]- exact mass	Elemental composition	Mass error (ppm)
Rha-C_{10}	333.19169	333.19188	$C_{16}H_{29}O_7$	0.57
Rha-Rha-C_{10}	479.24981	479.24979	$C_{22}H_{39}O_{11}$	-0.042
Rha-C_{10}-C_{10}	503.32234	503.32256	$C_{26}H_{47}O_9$	0.44
Rha-C_{10}-C_{12}[a]	531.35332	531.35386	$C_{28}H_{51}O_9$	1.02
Rha-Rha-C_{10}-C_8[b]	621.34942	621.34917	$C_{30}H_{53}O_{13}$	-0.4
Rha-Rha-C_{10}-C_{10}	649.38051	649.38047	$C_{32}H_{57}O_{13}$	-0.06
Rha-Rha-C_{10}-C_{12}[c]	677.41140	677.41177	$C_{34}H_{61}O_{13}$	0.55

[a] Or Rha-C_{12}-C_{10}

[b] Or Rha-Rha-C_8-C_{10}

[c] Or Rha-Rha-C_{12}-C_{10}

Table 2.5. Relative abundance (%) of rhamnolipid congeners produced by *Pseudomonas aeruginosa* #112 with the different culture media, in fermentations performed in flasks and reactor.

Rhamnolipid congener	**MW**	**Flask**			**Reactor**	
		CSLM	**CSLM + 15% OMW**	**CSLM + 25% OMW**	**CSLM**	**CSLM + 25% OMW**
Rha-C_{10}	334.19915	38%	32%	29%	38%	30%
Rha-C_{10}-C_{10}	504.32983	100%	100%	100%	100%	100%
Rha-C_{10}-C_{12}[a]	532.36113	12%	12%	13%	12%	12%
Rha-Rha-C_{10}	480.25706	12%	14%	11%	20%	10%
Rha-Rha-C_{10}-C_8[b]	622.35644	4%	4%	4%	14%	2%
Rha-Rha-C_{10}-C_{10}	650.38774	60%	66%	58%	78%	32%
Rha-Rha-C_{10}-C_{12}[c]	678.41904	14%	14%	12%	18%	16%
Ratio mono-/di-rhamnolipids	-	1.67	1.47	1.67	1.15	2.37

[a] Or Rha-C_{12}-C_{10}

[b] Or Rha-Rha-C_8-C_{10}

[c] Or Rha-Rha-C_{12}-C_{10}

In assays performed in reactor, using the medium CSLM supplemented with 25% of OMW, a lower relative abundance of all the di-rhamnolipid congeners, as well as the mono-rhamnolipid Rha-C_{10} was observed when compared with the medium CSLM, which resulted in a higher proportion of mono-rhamnolipids. It has been previously reported that the constituents of the culture medium exert a significant effect in the composition (both qualitative and quantitative) of the rhamnolipid mixtures produced by *P. aeruginosa* strains (Gudiña et al., 2015; Ismail et al., 2015; Zhang et al., 2014). *P. aeruginosa* strains produce rhamnolipids as combinations of different congeners (up to 60 different types have been reported), which can be mono- and di-rhamnolipids with one or two acyl chains containing 8, 10, 12 or 14 carbons, mostly saturated, and, less often, containing one or two double bonds, being the most common Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10} (Abdel-Mawgoud et al., 2010; Aparna et al., 2012; Benincasa and Accorsini, 2008; Bharal and Konwar, 2011; Haba et al., 2003; Ismail et al., 2015; Ramírez et al., 2015; Zhang et al., 2014). The size of the hydrophilic head (one or two rhamnoses) and the hydrophobic tail (one or two fatty acids), the length of the acyl chains and the presence of double bonds affect the surface-active properties of rhamnolipids. More hydrophilic congeners (di-rhamnolipids, mono-rhamnolipids with only one fatty acid, and congeners with shorter acyl chains) exhibit high *cmc* (up to 200 mg/L), whereas more hydrophobic ones (mono-rhamnolipids with two fatty acids and congeners with longer acyl chains) show low *cmc* (between 5 and 40 mg/L) and are more surface-active, reducing the surface tension more efficiently (Abdel-Mawgoud et al., 2009; Bharal and Konwar, 2011; Haba et al., 2003; Sarachat et al., 2010; Zhang et al., 2014). This could explain why the rhamnolipid mixtures produced in the media supplemented with OMW, which in general contain a higher proportion of more hydrophobic congeners, exhibited lower *cmc* when compared with those produced in the medium CSLM.

2.4. CONCLUSIONS

In this work, rhamnolipids production by a *P. aeruginosa* strain was demonstrated using a culture medium containing as sole ingredients the agro-industrial by-products CSL, molasses and OMW, which were used without any previous treatment. This low-cost culture medium allowed the production of 5.1 g of rhamnolipids per liter in reactor. Furthermore, the rhamnolipids produced exhibited a very low *cmc* (13 mg/L). This culture

medium represents a sustainable alternative for the production of rhamnolipids, and simultaneously it allows the valorization of the environmentally hazardous residue OMW.

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CHAPTER 3

SODIUM CHLORIDE EFFECT ON THE AGGREGATION BEHAVIOUR OF RHAMNOLIPIDS AND THEIR ANTIFUNGAL ACTIVITY

ABSTRACT

In this chapter, the antifungal activity of rhamnolipids produced by *Pseudomonas aeruginosa* #112 was evaluated against *Aspergillus niger* MUM 92.13 and *Aspergillus carbonarius* MUM 05.18. It was demonstrated that the di-rhamnolipid congeners were responsible for the antifungal activity exhibited by the crude rhamnolipid mixture, whereas mono-rhamnolipids showed a weak inhibitory activity. Furthermore, in the presence of NaCl (from 375 mM to 875 mM), the antifungal activity of the crude rhamnolipid mixture and the purified di-rhamnolipids was considerably increased. Dynamic Light Scattering studies showed that the size of the structures formed by the rhamnolipids increased as the NaCl concentration increased, being this effect more pronounced in the case of di-rhamnolipids. These results were confirmed by Confocal Scanning Laser Microscopy, which revealed the formation of giant vesicle-like structures (in the µm range) by self-assembling of the crude rhamnolipid mixture in the presence of 875 mM NaCl. In the case of the purified mono- and di-rhamnolipids, spherical structures (also in the µm range) were observed at the same conditions. The results herein obtained demonstrated a direct relationship between the rhamnolipids antifungal activity and their aggregation behavior, opening the possibility to improve their biological activities for application in different fields.

Keywords: Di-Rhamnolipids, Mono-Rhamnolipids, Antifungal activity, NaCl, Aggregation behavior

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CONTENTS

3.1. INTRODUCTION......83

3.2. MATERIAL AND METHODS......84

3.2.1. MICROORGANISMS AND CULTURE CONDITIONS......84

3.2.2. AGRO-INDUSTRIAL RESIDUES......85

3.2.3. RHAMNOLIPIDS PRODUCTION AND RECOVERY......85

3.2.4. PURIFICATION OF MONO-RHAMNOLIPIDS AND DI-RHAMNOLIPIDS......85

3.2.5. DETERMINATION OF CRITICAL MICELLE CONCENTRATION OF MONO- AND DI-RHAMNOLIPIDS......86

3.2.6. ANTIFUNGAL ACTIVITY ASSAYS......86

3.2.7. MICELLE SIZE MEASUREMENT BY DYNAMIC LIGHT SCATTERING (DLS)87

3.2.8. CONFOCAL SCANNING LASER MICROSCOPY......88

3.3. RESULTS AND DISCUSSION......88

3.3.1. PURIFICATION OF MONO-RHAMNOLIPIDS AND DI-RHAMNOLIPIDS CONGENERS PRODUCED BY *PSEUDOMONAS* #112......88

3.3.2. ANTIFUNGAL ACTIVITY OF THE RHAMNOLIPID MIXTURE PRODUCE BY *P. AERUGINOSA* #112......89

3.3.3. ANTIFUNGAL ACTIVITY OF MONO-RHAMNOLIPIDS AND DI-RHAMNOLIPIDS CONGENERS......92

3.3.4. EFFECT OF NACL ON THE SURFACE ACTIVITY AND THE AGGREGATION BEHAVIOR OF RHAMNOLIPIDS......94

3.4. CONCLUSIONS......98

3.5. REFERENCES......99

3.1. INTRODUCTION

Molds play an important role in the spoilage of agriculture products, especially during storage. Fungal contaminations can be disastrous to crops despite the preventive measures adopted for their control. Management of fungal contaminations is generally a difficult challenge, since once initiated, epidemics are very difficult to contain (Gerez et al., 2010; Mnif et al., 2015). It is estimated that 5 to 10% of the World's food production is lost due to fungal contamination, which causes high economic losses and several health risks due to the toxicity and pathogenic nature of some species (Bennett and Klich, 2003; Mnif et al., 2015). The extensive use of chemical fungicides to control plant diseases has a negative impact in the ecological balance of microorganisms inhabiting soil, due to the development of resistant strains of pathogens, groundwater contamination, and health risks to humans (Gerez et al., 2010; Pekmezovic et al., 2015). Species belonging to the genus *Aspergillus* are among the most common fungal contaminants, being some of them pathogenic for plants and animals (Ribes et al., 2017). Among them, *Aspergillus niger* and *Aspergillus carbonarius* are capable of growing in a wide range of organic substrates, causing deterioration of stored food material. Furthermore, *A. carbonarius* is one of the most important opportunistic pathogens of grapes (Lappa et al., 2017; Wu et al., 2014). The development of novel, more effective, safe and environmental friendly antifungal agents as an alternative to the chemical fungicides used for combating a variety of crop diseases is currently an environmental challenge (Mnif et al., 2015; Pekmezovic et al., 2015).

Several biosurfactants have been reported as effective biocontrol agents; however, only few reports describe rhamnolipids for this application. Rhamnolipids exhibit antagonistic activity against some fungi (Deepika et al., 2015; Kim et al., 2000; Nalini and Parthasarathi, 2014; Reddy et al., 2016; Yan et al., 2014) and have been demonstrated to inhibit pathogenic fungi resistant to commercial chemical pesticides (Sha et al., 2012). Moreover, rhamnolipids stimulate plant immunity, which can be considered as an alternative strategy to reduce the infections caused by plant pathogens (Mnif et al., 2015; Yan et al., 2014). Rhamnolipids are glycolipid biosurfactants produced mainly by *Pseudomonas* and *Burkholderia* species, being *Pseudomonas aeruginosa* the main producer (Geys et al., 2014; Kahraman and Erenler, 2012). The structure of rhamnolipids comprises one (mono-rhamnolipids) or two (di-rhamnolipids) rhamnose molecules linked to one or two β-hydroxy fatty acids which can differ in the chain length (8–24 carbons) and the degree of

saturation (0–2 double bonds) (Sha et al., 2012). Rhamnolipids are usually produced as a mixture of different congeners, and the ratio between mono-rhamnolipids and di-rhamnolipids depends on the bacterial strain, the culture medium composition and the operational conditions (Gudiña et al., 2016; Müller et al., 2012). Rhamnolipids can be synthesized using renewable low-cost substrates, such as plant oil and grain starch (Wang et al., 2007), wastes from sunflower oil (Benincasa and Accorsini, 2008), molasses (Al-Bahry et al., 2013; Gudiña et al., 2015), corn steep liquor (CSL) (Gudiña et al., 2015) and olive oil mill wastewater (OWM) (Gudiña et al., 2016), which could contribute to reduce their production costs. Due to their excellent surface activity, together with their environmentally-friendly nature, they can be used as wetting, sticker or dispersal agents for application in fungicides or pesticides. Indeed, rhamnolipids are already applied in the formulation of the commercial biofungicide ZonixTM (NOP Supply LLC, USA).

The aim of this chapter was to evaluate the antifungal activity of rhamnolipids produced by *P. aeruginosa* #112 against *A. niger* and *A. carbonarius*. A low-cost culture medium, containing molasses and CSL, was used for rhamnolipid production. Furthermore, it was studied which congeners (mono-rhamnolipids or di-rhamnolipids) were responsible for the antifungal activity observed. Finally, the relationship between the antifungal activity and the aggregation behavior of these rhamnolipids was studied.

3.2. MATERIAL AND METHODS

3.2.1. Microorganisms and culture conditions

The bacterial strain *P. aeruginosa* #112 was used for rhamnolipids production. This strain, isolated from a crude oil sample obtained from a Brazilian oil field, was previously reported as a promising rhamnolipid producer (Gudiña et al., 2015). It was stored at -80ºC in Luria Bertani (LB) medium supplemented with 20% (v/v) of glycerol. The composition of LB medium was (g/L): NaCl 10.0; tryptone 10.0; yeast extract 5.0; pH 7.0. The fungal strains *A. niger* MUM 92.13 and *A. carbonarius* MUM 05.18 were obtained from the culture collection of Micoteca of Universidade do Minho (MUM, Portugal). They were maintained at -80ºC in sterile demineralized water supplemented with 20% (v/v) of glycerol. Whenever required, frozen stocks of the fungal strains were streaked on Malt Extract Agar (MEA) plates and incubated at 25ºC for 7 days. The agar plates were stored

at 4ºC no longer than 3 weeks. The composition of MEA medium (Blakeslee's formula) was (g/L): malt extract 20.0; glucose 20.0; peptone 1.0; agar 20.0; pH 5.5.

3.2.2. Agro-industrial residues

Molasses and CSL were kindly provided by RAR: Refinarias de Açúcar Reunidas, S.A. (Portugal) and COPAM: Companhia Portuguesa de Amidos, S.A. (Portugal), respectively. The composition of these residues was reported in Chapter 2.

3.2.3. Rhamnolipids production and recovery.

The production of rhamnolipids by *P. aeruginosa* #112 was performed in 1000 mL flasks containing 400 mL of the culture medium CSLM (Gudiña et al., 2015) (molasses (10% (w/v)); CSL (10% (v/v)); pH 7.0). Each flask was inoculated with 1% (v/v) of a pre-culture of *P. aeruginosa* #112 grown for 24 h in LB medium at 37ºC and 180 rpm. Subsequently, the flasks were incubated at the same conditions for 120 h. Rhamnolipids production was monitored along the fermentation by measuring the surface tension of the cell-free supernatants obtained after centrifuging (10 000 × g, 20 min, 20ºC) the samples recovered at different time points as described in Chapter 2. At the end of the fermentation, the cultures were centrifuged (10 000 × g, 20 min, 20ºC), and the rhamnolipids produced were recovered from the cell-free supernatants by adsorption chromatography using the polystyrene resin Amberlite XAD-2 (Sigma-Aldrich Co., USA) as described in Chapter 2. Rhamnolipids production was determined as the dry weight of the freeze-dried product (crude rhamnolipid mixture).

3.2.4. Purification of mono-rhamnolipids and di-rhamnolipids

The mono-rhamnolipids and di-rhamnolipids congeners present in the crude rhamnolipids mixture were separated and purified through silica gel column chromatography. A 200 mL glass column was filled with silica gel 60 (particle size 63–200 µm, mesh size 70–230 (Sigma-Aldrich Co., USA)) and equilibrated with 100 mL of chloroform at a constant flow rate of 1 mL/min. 500 mg of the freeze-dried crude rhamnolipid mixture were dissolved in 50 mL of chloroform and loaded onto the column. Subsequently, the column was washed with 100 mL of chloroform to remove the non-

adsorbed compounds (i.e. neutral lipids). After that, the mono-rhamnolipids and di-rhamnolipids congeners were separated by elution with chloroform:methanol mixtures with increasing polarity: 180:5 (v/v) for mono-rhamnolipids and 180:20 (v/v) for di-rhamnolipids. All the steps were performed at the same flow rate (1 mL/min). Finally, the solvents were evaporated and the purified mono-rhamnolipids and di-rhamnolipids fractions were dissolved in a minimal amount of demineralized water and freeze-dried. The products obtained were weighed and stored at −20ºC for further studies. The purification process was monitored through thin layer chromatography (TLC). Samples of the different fractions recovered were spotted onto silica gel TLC plates (DC-Fertigfolien ALUGRAMR SIL G UV_{254}, Macherey-Nagel GmbH & Co., Germany) which were developed using a solvent system consisting of chloroform: methanol:water (65:25:4, v/v/v). The isolated compounds on the TLC plate were located by spraying it with a solution containing orcinol (0.19% (w/v)) in 53% (w/v) sulphuric acid, followed by incubation at 105ºC until the plots became visible. A sample of the crude rhamnolipids mixture (containing mono-rhamnolipids and di-rhamnolipids) was used as reference.

3.2.5. Determination of critical micelle concentration of mono- and di-rhamnolipids

The critical micelle concentrations (*cmc*) of the purified mono-rhamnolipid and di-rhamnolipid fractions were calculated as described in the previous chapter (Chapter 2), using the freeze-dried samples dissolved in demineralized water at different concentrations. All the measurements were performed in triplicate.

3.2.6. Antifungal activity assays

A. niger MUM 92.13 and *A. carbonarius* MUM 05.18 were cultured in MEA plates at 25ºC for 7 days. To prepare the spore suspensions, the fungus surface was washed with 1 mL of sterile demineralized water, which was subsequently transferred to a sterile tube. The spore suspensions were diluted with sterile demineralized water to a concentration of 10^5 spores/mL and stored at 4ºC no longer than 3 weeks. The spores were counted using a Neubauer improved cell counter (Marienfeld GmbH, Germany). The antifungal activity was studied using the cell-free supernatant obtained at the end of the fermentations performed with *P. aeruginosa* #112, the crude rhamnolipid mixture, and the purified mono-

rhamnolipids and di-rhamnolipids. The assays were performed in Petri dishes (55 mm diameter) containing MEA medium. For the crude rhamnolipid mixture and the purified mono-rhamnolipids and di-rhamnolipids, the freeze-dried products were added to the culture medium at different concentrations. When the cell-free supernatant was evaluated, it was used to dissolve the components of the MEA medium instead demineralized water. In all the cases, the pH of the culture medium was adjusted to 5.5. All the media were autoclaved at 121ºC for 15 min. The agar plates were inoculated with 10 µL of the corresponding spore suspension in the center of the plate. Subsequently, the plates were incubated at 25ºC for 5 days. The fungal growth was determined by measuring the diameter of the growth zone. The percentage of growth inhibition relative to the control was calculated as follows:

$$\text{(3.1)} \qquad Growth\ inhibition\ x\ (\%) = \left(1 - \frac{diameter\ x}{diameter\ c}\right) \times 100$$

where *diameter x* (cm) represents the diameter of the mycelial growth in the medium with the treatment *x*, and *diameter c* represents the diameter of the fungal growth in the corresponding control. All the experiments were performed in triplicates.

3.2.7. Micelle size measurement by dynamic light scattering (DLS)

The size distribution and the polydispersity indexes (PDI) of the assembled structures formed by the rhamnolipids under study (crude rhamnolipid mixture and purified mono-rhamnolipids and di-rhamnolipids) dissolved in ultrapure water at different concentrations were measured by DLS using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., UK) at 25ºC. The refractive index of the samples was found to be 1.330, and the scattering angle was 173º. The PDI qualifies the particle size distribution, which here ranged from 0 for monodispersed to 1 for entirely heterodispersed samples. Each sample was analyzed ten times, and information about size distribution by intensity was recorded and averaged.

3.2.8. Confocal scanning laser microscopy

A Confocal Scanning Laser Microscope (Olympus BX61, Model Fluo View 1000) was used to visualize the structures formed by the crude rhamnolipid mixture and the mono-rhamnolipid and di-rhamnolipid congeners in different aqueous solutions using Nile Red (Sigma-Aldrich Co., USA) as fluorescence probe. Nile Red and rhamnolipid solutions were prepared in ethanol at a concentration of 1 mg/mL; subsequently both solutions were mixed (700 μL of rhamnolipids and 1 μL of Nile Red) and incubated at 150 rpm at room temperature for 30 min. After that, the ethanol was evaporated, and the samples were dissolved in 100 μL of demineralized water or 875 mM NaCl. The samples were immediately observed using the laser excitation line at 559 nm in combination with emission filters BA 575–675. The images were acquired using the program FV10 (Version 4.1.1.5, Olympus).

3.3. RESULTS AND DISCUSSION

3.3.1. Purification of mono-rhamnolipids and di-rhamnolipids congeners produced by *Pseudomonas* #112.

P. aeruginosa #112 was previously reported to produce eight different rhamnolipid congeners using the culture medium CSLM. Among them, the most abundant were the mono-rhamnolipid Rha-C_{10}-C_{10} and the di-rhamnolipid Rha-Rha-C_{10}-C_{10}, although lower amounts of other congeners with acyl chains containing 8 or 12 carbons were also identified (Gudiña et al., 2015). The mono-rhamnolipid and di-rhamnolipid congeners present in the crude rhamnolipid mixture were separated through silica gel column chromatography. Two different fractions were obtained, and these were analyzed through TLC in order to confirm the presence of mono-rhamnolipids or di-rhamnolipids. As expected, each fraction exhibited a single spot, which Rf values were approximately 0.70 and 0.47 for the mono-rhamnolipid and di-rhamnolipid samples, respectively. These results are in accordance with previous reports (Abdel-Mawgoud et al., 2009; HoŠková et al., 2013; Sha et al., 2012). After the separation process, 500 mg of the crude rhamnolipid mixture yielded 51.7 ± 7.6 mg of mono-rhamnolipids and 149.0 ± 48.9 mg of di-rhamnolipids. According to the results obtained, only 40% of the crude rhamnolipid sample corresponds to rhamnolipids, thus it can be assumed that the remaining 60% were impurities.

3.3.2. Antifungal activity of the rhamnolipid mixture produce by *P. aeruginosa* #112

In a first approach, the antifungal activity of the rhamnolipids produced by *P. aeruginosa* #112 against the fungi *A. niger* MUM 92.13 and *A. carbonarius* MUM 05.18 was evaluated using the cell-free supernatant obtained at the end of the fermentation performed in the culture medium CSLM, which contains about 3 g of rhamnolipids per liter (Gudiña et al., 2015). The cell-free supernatant completely inhibited the growth of *A. carbonarius* MUM 05.18, whereas in the case of *A. niger* MUM 92.13, 75.5 ± 2.7% of growth inhibition was achieved. These results are in good agreement with previous studies that reported the antagonistic activity of rhamnolipids against other genera of fungi, including *Botrytis*, *Colletotrichum*, *Cylindrocarpon*, *Fusarium*, *Magnaporthe*, *Mucor*, *Phytophthora* and *Macrophomina* (Kim et al., 2000; Nalini and Parthasarathi, 2014; Reddy et al., 2016; Sha et al., 2012). Although the mechanisms responsible for this antifungal activity are not well established, it is assumed that low molecular weight biosurfactants like rhamnolipids interact with the lipid constituents of biological membranes, disturbing their integrity and permeability by inducing the formation of pores and ion channels (Mnif et al., 2015; Sánchez et al., 2007). Subsequently, the antifungal activity was studied using the partially purified crude rhamnolipid mixture at different concentrations (0.375–12 g/L). Surprisingly, the growth inhibition percentages obtained for a rhamnolipid concentration of 3 g/L were considerably lower (28.0 ± 8.6% for *A. niger* MUM 92.13 and 22.6 ± 1.2% for *A. carbonarius* MUM 05.18) when compared with those achieved with the cell-free supernatant. Even using a crude rhamnolipid concentration of 12 g/L, low growth inhibition percentages (between 28 and 30%) were found. Similarly, to the results herein obtained, Sha and co-workers (2012) reported that the cell-free supernatant containing rhamnolipids exhibited a higher antifungal activity against several plant pathogens when compared with the purified mono-rhamnolipid and di-rhamnolipid congeners. In this case, the authors suggested that this could be due to the presence of both types of rhamnolipids in the cell-free supernatant. Haba and co-workers (2003) also reported that a purified mixture of mono-rhamnolipid and di-rhamnolipid congeners exhibited a weak inhibitory activity against several yeast and fungal strains (including *A. niger*), although it showed a considerable antimicrobial activity against several Gram-positive and Gram-negative bacteria. Due to the discrepancy observed between the antifungal activities exhibited by the cell-free supernatant and the crude rhamnolipid mixture, further studies were performed to

establish if other compounds (instead the rhamnolipids) present in the culture medium CSLM or in the cell-free supernatant were responsible for the antifungal activity. These studies were performed in the same way that those previously described but replacing the cell-free supernatant containing rhamnolipids by the culture medium CSLM or by the cell-free supernatant after removing the rhamnolipids. In none of these studies was observed antifungal activity, suggesting that it must be due to the rhamnolipids. It has been previously reported that the biological activity of rhamnolipids is related to their aggregation behavior, which is affected by the pH and the presence of electrolytes (Champion et al., 1995; Otzen, 2016). In order to validate this hypothesis, the antifungal activity of the crude rhamnolipid mixture was studied in the presence of different NaCl concentrations. Control assays were performed using the culture medium MEA supplemented with the same NaCl concentrations tested (250–1000 mM), and it was found that they did not inhibit the fungal growth. On the contrary, in the case of *A. niger* MUM 92.13, the addition of NaCl into the culture medium at concentrations between 750 and 1000 mM enhanced the fungal growth by 8.9 and 19.8%, respectively. It was found that, for both fungi, the addition of NaCl to the culture medium increased the antifungal activity of the crude rhamnolipid mixture. The lowest NaCl concentrations that resulted in a complete growth inhibition in combination with the crude rhamnolipid mixture (3 g/L) were 875 mM for *A. niger* MUM 92.13 (Table 3.1) and 375 mM for *A. carbonarius* MUM 05.18 (Table 3.2). For the other rhamnolipid concentrations tested (0.375, 0.75 and 1.5 g/L), the addition of NaCl (875 mM for *A. niger* MUM 92.13 and 375 mM for *A. carbonarius* MUM 05.18) to the culture medium also increased the antifungal activity when compared with the assays performed without NaCl (Table 3.1 and Table 3.2). The explanation for this behavior could be that the combination of NaCl with the crude rhamnolipid mixture allowed the formation of structures similar to those originally present in the cell-free supernatant, which confer the antifungal activity to the rhamnolipids. This organization must be lost during the recovery of the rhamnolipids, which resulted in a considerable reduction of their antifungal activity.

Table 3.1. Growth inhibition percentages obtained for *Aspergillus niger* MUM 92.13 with crude rhamnolipid mixture produce by *Pseudomonas aeruginosa* #112. The assays preformed at different NaCl concentrations, at 25℃ for 5 days. The results represent the average of the three independent experiments ± standard deviation. [RL]: concentration of crude rhamnolipid mixture. NT: not tested.

	[NaCl] (mM)				
	0	**500**	**750**	**875**	**1000**
[RL] g/L	**Growth inhibition (%)**				
0.375	17.1 ± 6.5	NT	NT	43.3 ± 1.2	NT
0.75	8.1 ± 3.7	NT	NT	53.2 ± 0.0	NT
1.5	13.0 ± 3.7	NT	NT	51.8 ± 1.2	NT
3.0	28.0 ± 8.6	29.1 ± 1.2	73.0 ± 1.1	100.0 ± 0.0	100.0 ± 0.0

Table 3.2. Growth inhibition percentages obtained for Aspergillus carbonarius MUM 05.18 with crude rhamnolipid mixture produce by Pseudomonas aeruginosa #112. The assays preformed at different NaCl concentrations, at 25℃ for 5 days. The results represent the average of the three independent experiments ± standard deviation. [RL]: concentration of crude rhamnolipid mixture. NT: not tested.

	[NaCl] (mM)			
	0	**250**	**375**	**500**
[RL] g/L	**Growth inhibition (%)**			
0.375	24.0 ± 2.1	NT	50.9 ± 6.5	NT
0.75	26.7 ± 10.3	NT	47.2 ± 3.8	NT
1.5	29.5 ± 8.3	NT	58.5 ± 3.8	NT
3.0	22.6 ± 1.2	78.0 ± 1.1	100.0 ± 0.0	100.0 ± 0.0

3.3.3. Antifungal activity of mono-rhamnolipids and di-rhamnolipids congeners.

The antifungal activity of the purified mono-rhamnolipid and di-rhamnolipid congeners against *A. niger* MUM 92.13 and *A. carbonarius* MUM 05.18 was studied at different concentrations (0.75–1.5 g/L for mono-rhamnolipids and 0.05–1.5 g/L for di-rhamnolipids), with and without NaCl, in order to evaluate their individual contribution. As it can be seen from the results obtained (Table 3.3 and Table 3.4), the di-rhamnolipid congeners were responsible for the antifungal activity observed with the crude rhamnolipid mixture, whereas the mono-rhamnolipids exhibited a weak inhibitory activity even at the highest concentration tested (1.5 g/L). As in the case of the crude rhamnolipid mixture, the antifungal activity exhibited by the di-rhamnolipid congeners was enhanced by the addition of NaCl. The purified di-rhamnolipids completely inhibited the growth of *A. niger* MUM 92.13 and *A. carbonarius* MUM 05.18 at concentrations of 0.2 and 0.75 g/L, respectively, but only when supplemented with the optimum NaCl concentrations (Table 3.3 and Table 3.4). Previous studies suggested that the biological activity associated to rhamnolipids is due to the di-rhamnolipid congeners. Sha and co-workers (2012) reported that di-rhamnolipids exhibit a higher antifungal activity against different plant pathogens when compared with mono-rhamnolipids, which exhibit only a weak inhibitory activity. Likewise, it was reported that mono-rhamnolipids inhibit the growth of *A. niger* only at very high concentrations (68 g/L) (Johann et al., 2016).

Table 3.3. Growth inhibition percentages obtained for *Aspergillus niger* MUM 92.13 with the purified mono-rhamnolipid and di-rhamnolipid congeners. The assays were performed at different of purified rhamnolipid congeners with and without NaCl, at 25ºC for 5 days. The results represent the average of three independent experiments ± standard deviation. [RL]: concentration of mono-rhamnolipid or di-rhamnolipid congeners. NT: not tested.

	Growth inhibition (%)			
[RL] g/L	**Mono-RL**	**Mono-RL + 875 mM NaCl**	**Di-RL**	**Di-RL + 875 mM NaCl**
0.05	NT	NT	NT	52.4 ± 1.2
0.1	NT	NT	NT	61.9 ± 1.2
0.2	NT	NT	NT	100.0 ± 0.0
0.375	NT	NT	41.0 ± 1.5	100.0 ± 0.0
0.75	NT	78.0 ± 1.1	NT	100.0 ± 0.0
1.5	46.2 ± 0.0	78.0 ± 1.1	40.0 ± 0.75	NT

Table 3.4. Growth inhibition percentages obtained for Aspergillus carbonarius MUM 05.18 with the purified mono-rhamnolipid and di-rhamnolipid congeners. The assays were performed at different of purified rhamnolipid congeners with and without NaCl, at 25ºC for 5 days. The results represent the average of three independent experiments ± standard deviation. [RL]: concentration of mono-rhamnolipid or di-rhamnolipid congeners. NT: not tested.

[RL] g/L	Growth inhibition (%)			
	Mono-RL	**Mono-RL + 375 mM NaCl**	**Di-RL**	**Di-RL +375 mM NaCl**
0.375	NT	NT	34.9 ± 1.3	72.6 ± 1.3
0.5	NT	NT	NT	80.7 ± 1.2
0.6	NT	NT	NT	73.8 ± 1.2
0. 75	NT	25.2 ± 4.4	NT	100.0 ± 0.0
1.5	30.2 ± 5.3	26.4 ± 2.7	33.1 ± 3.2	NT

One of the mechanisms proposed for the biological activities of biosurfactants is their interaction with the phospholipid bilayer, which results in a detergent-like effect that disrupts the plasma membrane (Akiyode et al., 2016). This interaction may be influenced by the composition of the hydrophilic head and hydrophobic tails of biosurfactants. It has been proposed that the large polar head group of di-rhamnolipids confers them an inverted-cone shape, which induces a positive curvature to the membranes and might be responsible for their disrupting effect (Inès and Dhouha, 2015; Otzen, 2016). However, contrary to our results, other authors reported that mono-rhamnolipids exhibit a higher antimicrobial activity against Gram-positive and Gram-negative bacteria when compared with di-rhamnolipids (Das et al., 2014). Christova and co-workers (2013) also reported that the mono-rhamnolipid congener Rha-C_{10}-C_{10} showed a higher cytotoxic activity against different human cancer cell lines when compared with the di-rhamnolipid congener Rha-Rha-C_{10}-C_{10}.

3.3.4. Effect of NaCl on the surface activity and the aggregation behavior of rhamnolipids.

The purified mono-rhamnolipids and di-rhamnolipids dissolved in demineralized water (500 mg/L) reduced the surface tension up to 25.9 ± 0.2 mN/m (*cmc* 50 mg/L) and 33.5 ± 0.3 mN/m (*cmc* 15 mg/L), respectively. The higher surface activity of mono-rhamnolipids when compared with di-rhamnolipids is attributed to their less hydrophilic character, which has been extensively reported in the literature (Das et al., 2014; Gudiña et al., 2015; Helvaci et al., 2004). Rhamnolipids have one or two hydrophobic chains, whereas their hydrophilic moieties are the rhamnosyl and the carboxylic groups. Mono-rhamnolipids have a single rhamnosyl group, whereas di-rhamnolipids have two. The rhamnosyl groups endow hydrophilicity to rhamnolipids, while the carboxylic groups carry out the functional control of their amphipathic properties, which are strongly affected by the pH and the presence of electrolytes (Helvaci et al., 2004). According to the results herein obtained, higher *cmc* values have been reported for mono-rhamnolipids (0.050 mM, ≈25 mg/L) when compared with di-rhamnolipids (0.010 mM, ≈6.5 mg/L) by other authors (Abbasi et al., 2013; Sánchez et al., 2007). Subsequently, it was studied the effect of NaCl on the surface tension and the *cmc* values. In the case of mono-rhamnolipids, the addition of NaCl (375 mM and 875 mM) did not have effect on the surface tension values but resulted in a considerable decrease of the *cmc* (25 mg/L) in both cases. In the case of di-rhamnolipids, a slight decrease in the

surface tension was observed (31.7 ± 0.1 mN/m) with the highest NaCl concentration tested (875 mM), but the *cmc* value remained constant. The *pKa* values reported for mono-rhamnolipids and di-rhamnolipids are 5.9 and 5.6, respectively (Abbasi et al., 2013; Sánchez et al., 2007). The pH of the rhamnolipid solutions herein used was not adjusted, and it was around 6.2. In these conditions, in the absence of an electrolyte, the majority of the carboxylic groups of rhamnolipids are dissociated to form carboxylate ions. As a result, the liquid surface exhibits a net negative charge, resulting in strong repulsive electrostatic forces between the rhamnolipid molecules. In the presence of NaCl, this negative charge is shielded by the Na^+ ions, causing the formation of a close-packed monolayer, which should result in a decrease in the *cmc* and the surface tension values (Abbasi et al., 2013; Helvaci et al., 2004; Sánchez et al., 2007). This compaction must be higher in the case of mono-rhamnolipids when compared with di-rhamnolipids, due to the larger di-rhamnose head group, which impose higher packing constraints (Chen et al., 2010; Helvaci et al., 2004). Although in this case the surface tension values were not affected by the addition of NaCl, this could explain why the *cmc* value of mono-rhamnolipids was reduced in the presence of NaCl, whereas in the case of di-rhamnolipids it was not affected. On the contrary, other authors proposed that rhamnolipids exhibit a weakly ionic nature, even at high pH values, which means that their surface tension and *cmc* values are not affected by the presence of electrolytes (Chen et al., 2013; Chen et al., 2010). Biosurfactants can self-assemble into a wide variety of morphologically different structures, including micelles, vesicles, multilamellar vesicles, lamellar structures or non-organized multilayers, among others (Rodrigues, 2015). It has been reported that the different aggregation behaviors are related with the biological activities exhibited by biosurfactants (Janek et al., 2016; Sánchez et al., 2007). In order to establish a relationship between the organization of rhamnolipids and the antifungal activities observed, the micellar size distribution of rhamnolipids in aqueous solution was studied at different NaCl concentrations through DLS, using the crude rhamnolipid mixture, as well as the purified mono-rhamnolipid and di-rhamnolipid fractions. The rhamnolipid concentrations used to perform these assays were selected according to the quality of the measurements (determined by the correlation function and the polydispersity index (PDI) value). According to the results obtained (Table 3.5), the purified mono-rhamnolipids and di-rhamnolipids formed micelles of similar size (130–140 nm) when dissolved in ultrapure water, whereas the crude rhamnolipid mixture formed micelles around 300 nm. The size of the structures formed by the rhamnolipids increased in all the cases as the NaCl concentration increased. This increase was higher for the

purified mono-rhamnolipids when compared with the crude rhamnolipid mixture. However, the most significant increase was observed for the di-rhamnolipid congeners; in this case it was not possible to measure the size of the structures formed for any of the NaCl concentrations tested, as they were out of the range of the equipment (10 µm).

Table 3.5. Effect of NaCl on the micellar size distribution of the crude rhamnolipid mixture and the mono-rhamnolipid and di-rhamnolipid congeners determined by DLS analysis. The concentrations of the crude rhamnolipid mixture, mono-rhamnolipids and di-rhamnolipids were 1.5, 1.0 and 0.5 g/L, respectively. The results represent the average of 10 measurements ± standard deviation. ND: not determined.

Rhamnolipid	[RL] (g/L)	NaCl (M)	Size (nm)	PDI
Crude (Mixture)	1.5	0.0	302.8 ± 7.4	0.549 ±0.009
		0.375	456.6 ± 42.2	0.596 ± 0.106
		0.875	2343 ± 154.1	0.753 ± 0.190
Mono-Rhamnolipid	1.0	0.0	140.3 ± 2.0	0.263 ± 0.006
		0.375	2212 ± 444.1	0.890 ± 0.107
		0.875	4674 ± 359.8	1.000 ± 0.000
Di-Rhamnolipid	0.5	0.0	133.1 ± 4.9	0.373 ± 0.042
		0.375	> 10 000	-
		0.875	> 10 000	-

The organization of the crude rhamnolipid mixture and the mono-rhamnolipid and di-rhamnolipid congeners in aqueous solution was analyzed through confocal scanning laser microscopy (Figure 3.1). The images revealed the formation of giant vesicle-like structures (in the µm range) by self-assembling of the crude rhamnolipid mixture in the presence of 875 mM NaCl. In the case of the purified mono-rhamnolipids and di-rhamnolipids, spherical structures were observed at the same conditions. The size of the structures formed

was in accordance with the results predicted by the DLS measurements. The images also confirmed the heterogeneity of the samples as predicted by the high PDI values (Table 3.5).

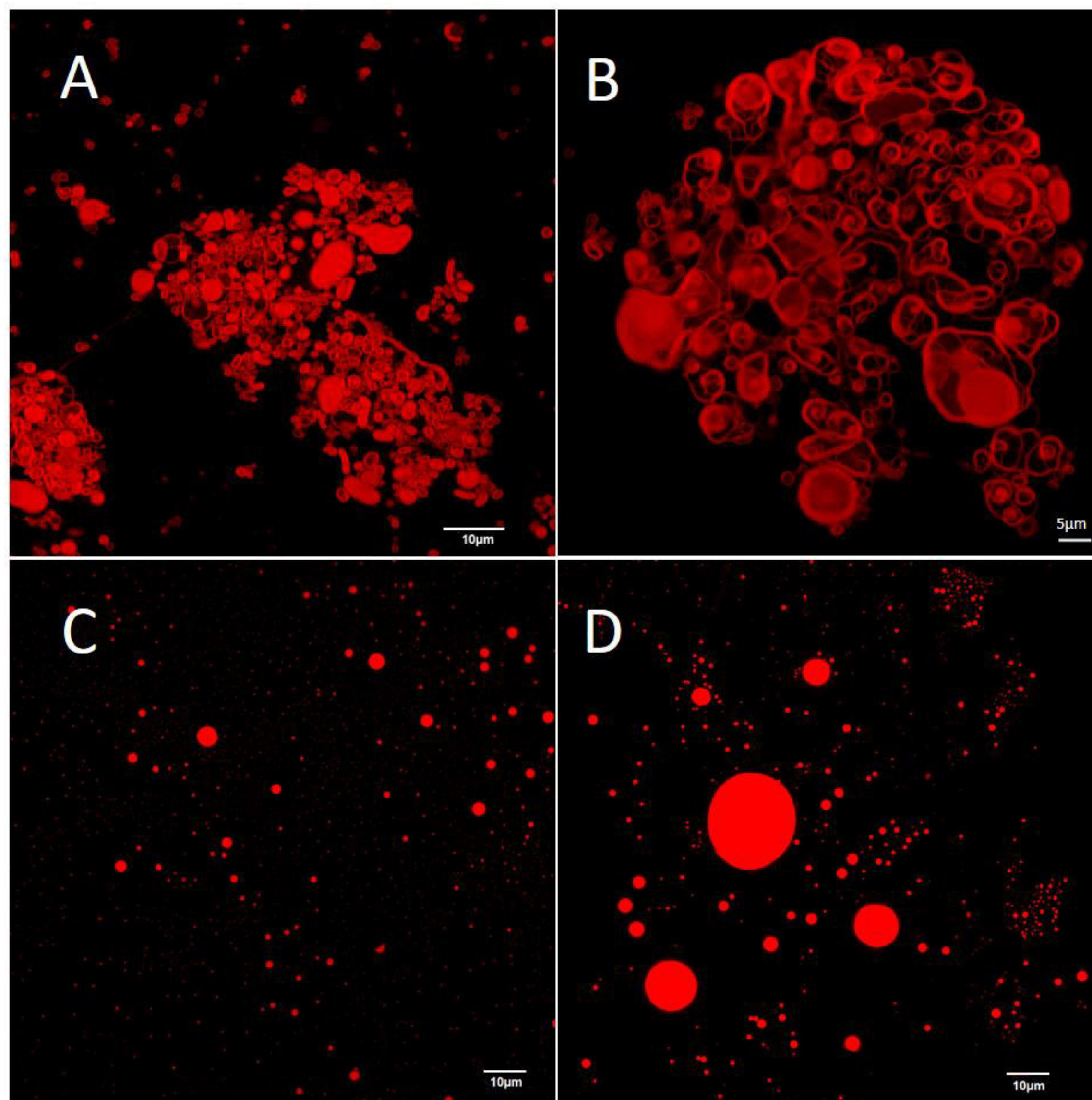

Figure 3.1 Confocal scanning laser microscopy images showing the self-assembled structures formed by crude rhamnolipid mixture (A, B), mono-rhamnolipids (C) and di-rhamnolipids (D) in the presence of 875 mM NaCl. Nile red was used as fluorescence probe.

None of these structures was observed in the absence of NaCl (*data not shown*). Consequently, it can be suggested that the changes observed in the aggregation behavior of rhamnolipids in the presence of NaCl are responsible for the increase in their antifungal activity against *A. niger* MUM 92.13 and *A. carbonarius* MUM 05.18. It has been

previously reported that the addition of divalent cations to anionic surfactant solutions promoted their micellar growth and a transition from micellar to lamellar structures (Chen et al., 2013; Chen et al., 2008; Janek et al., 2016). In the case of rhamnolipids, the NaCl concentration exhibits a significant effect on the size of the rhamnolipid aggregates, and it has been reported the formation of spherical micelles, lamellar vesicles, bilayers and rod shaped micelles as a function of the NaCl concentration (Champion et al., 1995; Helvaci et al., 2004), as well as the transition from micellar to lamellar structures as a result of the addition of electrolytes (Chen et al., 2010). In the case of di-rhamnolipids, the formation of non-micellar aggregates and lamellar vesicles (with sizes higher than 1500 nm) in the presence of 150 mM NaCl has been reported by other authors, but only at high di-rhamnolipid concentrations (2.5 mM, ≈1.6 g/L), whereas at lower di-rhamnolipid concentrations (1 mM, ≈0.65 g/L), micelles (43–66 nm) and non-micellar aggregates (350–550 nm) coexist in similar proportions (Sánchez et al., 2007). On the contrary, in the case of mono-rhamnolipids, even at high concentrations (up to 3 mM, ≈1.5 g/L), the formation of aggregates with a lower average size (200–210 nm) has been reported in the presence of 100 mM NaCl (Abbasi et al., 2013). Micelle-to-vesicle transition was only observed at a very high mono-rhamnolipid concentration (50–100 mM, ≈25–50 g/L) (Chen et al., 2010). In our case, the size of the structures formed by the crude rhamnolipid mixture and the purified mono-rhamnolipids and di-rhamnolipids was higher when compared with those reported by other authors. However, the formation of vesicle-like structures was only observed for the crude rhamnolipid mixture, but not for the purified mono-rhamnolipids and di-rhamnolipids.

3.4. CONCLUSIONS

Rhamnolipids produced by *P. aeruginosa* #112 in the culture medium CSLM exhibited antifungal activity against *A. niger* and *A. carbonarius* strains, being a promising alternative to the chemical fungicides commonly used. It was demonstrated that the di-rhamnolipid congeners are responsible for the antifungal activity observed, whereas the mono-rhamnolipids exhibited only a weak inhibitory activity. It was also observed that the antifungal activity is lost during the recovery of the rhamnolipids from the culture medium, and it was restored by the addition of NaCl, which also altered their aggregation behavior. Consequently, a relationship between the antifungal activity of rhamnolipids and their

aggregation behavior was observed, which can be useful for the development of more effective antifungal agents with better and improved properties.

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CHAPTER 4

RHAMNOLIPIDS PRODUCED BY *PSEUDOMONAS AERUGINOSA* AND *BURKHOLDERIA THAILANDENSIS* INHIBIT AFLATOXINS PRODUCTION BY *ASPERGILLUS FLAVUS*

ABSTRACT

In this chapter, the effect of rhamnolipids produced by *Pseudomonas aeruginosa* #112 and *Burkholderia thailandensis* E264 on the growth and aflatoxins production by *Aspergillus flavus* MUM 17.14 was evaluated. Aflatoxin production was inhibited by 99.5-99.8% by both rhamnolipid mixtures at a concentration of 1500 mg/L. However, at low concentrations (45-90 mg/L), the crude rhamnolipids produced by *P. aeruginosa* exhibited a higher inhibition (94%) compared to those from *B. thailandensis* (56-65%) due to the synergistic effect of the combination of mono- and di-rhamnolipids. This was demonstrated by studying the effect of the different rhamnolipid congeners on the aflatoxin production. Regarding the effect on the growth of *A. flavus* MUM 17.14, the rhamnolipids produced by *P. aeruginosa* exhibited a higher growth inhibition (34-40%) than the ones from *B. thailandensis* (4-7%). This difference is most probably due to the different length of the fatty acid chains of rhamnolipids produced by both microorganisms. Overall, the results herein gathered demonstrate for the first time that rhamnolipids are promising candidates for application as antagonistic compounds against aflatoxigenic fungi, although their activity is dependent on the type of congener and their relative proportions.

Keywords: Aflatoxins, *Aspergillus flavus*, Rhamnolipids, *Pseudomonas aeruginosa*, *Burkholderia thailandensis*

CONTENTS

4.1. INTRODUCTION......106

4.2. MATERIALS AND METHODS......108

4.2.1. MICROORGANISMS AND CULTURE CONDITIONS......108

4.2.2. AGRO-INDUSTRIAL RESIDUES......108

4.2.3. RHAMNOLIPIDS PRODUCTION, RECOVERY AND PURIFICATION......108

4.2.4. EFFECT OF RHAMNOLIPIDS ON AFLATOXINS PRODUCTION......109

4.2.5. STATISTICAL ANALYSIS......110

4.3. RESULTS AND DISCUSSION......111

4.3.1. ANTIFUNGAL ACTIVITY OF RHAMNOLIPIDS AGAINST *A. FLAVUS* MUM 17.14…… 111

4.3.2. EFFECT OF RHAMNOLIPIDS ON AFLATOXINS PRODUCTION......115

4.4. CONCLUSIONS......118

4.5. REFERENCES......119

4.1. INTRODUCTION

The contamination of foodstuffs and agricultural commodities by mycotoxin-producing fungi is a severe problem that causes substantial economic and health impacts due to losses in crop yield and quality, as well as their toxicity to humans and animals. Mycotoxins are a heterogeneous group of extremely toxic secondary metabolites synthesized by certain filamentous fungi that exhibit carcinogenic, mutagenic and teratogenic activities (Barrett, 2000). The major cause of human exposure to mycotoxins is the consumption of contaminated crops or foods prepared from them. For instance, it is estimated that approximately 25% of the cereals produced worldwide are contaminated with mycotoxins (Lee and Ryu, 2017). Consequently, several countries have established maximum acceptable concentrations for specific mycotoxins in food and feed, although the permissible threshold levels vary widely in different countries and different products (Barrett, 2000; Divakara et al., 2015; Simas et al., 2007; Yu et al., 2004).

Species belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium* are among the most frequently reported agents of food and feed contamination, and also the primary source of mycotoxins in a wide variety of crops. Aflatoxins are one of the most relevant groups of mycotoxins, being *Aspergillus flavus* the main producer (Divakara et al., 2015). Among the different types, aflatoxin B_1 (AFB_1) is considered the most toxic aflatoxin and is classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC, 2002).

The presence of mycotoxins in food and feed, even at low concentrations, can cause serious health problems as a result of their accumulation, due to the consumption of mycotoxin-contaminated products over a long time period (Bennett and Klich, 2003; Simas et al., 2007). Therefore, the levels of mycotoxins in such products should be reduced to the lowest technologically possible. Accordingly, several strategies (including chemical, physical and biological methods) have been investigated to reduce fungal and mycotoxins contamination in crops (Barrett, 2000; EFSA, 2009; Simas et al., 2007; Veras et al., 2016). However, such methods are usually limited by their partial efficiency or the alteration of food quality parameters (Hernández-Falcón et al., 2018). Therefore, the prevention of mycotoxins synthesis remains the best way to avoid food contamination. The use of synthetic fungicides may be a useful strategy to control pests, fungal contamination and postharvest diseases in crops. However, these agents must be completely lethal, otherwise

they may stimulate the production of mycotoxins, or their constant use can result in the development of fungal resistance if there is no good manufacturing practice (Gareis and Ceynowa, 1994; Mossini et al., 2009). Moreover, there is a great need to find innovative solutions for controlling these pathogens due to the negative impact of chemical fungicides on the human health and environment (Sha et al., 2012).

In that sense, biological control appears to be the most promising approach to manage mycotoxins contamination in both pre- and post-harvested crops. The applicability of antagonistic bacterial strains for this purpose has been widely demonstrated in the last years. Among them, *Bacillus* species which produce antibiotics (including lipopeptide biosurfactants) that inhibit toxigenic fungi are among the most remarkable biocontrol agents. Lipopeptide biosurfactants, due to their ability of reducing the fungal growth and the production of mycotoxins, could be used as biopesticides for plant and post-harvest protection (González Pereyra et al., 2018; Veras et al., 2016). Furthermore, these compounds are easily biodegradable in soils, being a healthier and environmentally-friendly alternative to the synthetic fungicides (Veras et al., 2016).

Rhamnolipids are a class of glycolipid biosurfactants produced by different microorganisms, including species belonging to the genera *Pseudomonas*, *Burkholderia*, *Acinetobacter* and *Enterobacter* (Abdel-Mawgoud et al., 2010; Dubeau et al., 2009). Rhamnolipids comprise a hydrophilic moiety, consisting of one (mono-rhamnolipids) or two (di-rhamnolipids) rhamnose molecules, linked to a hydrophobic moiety, consisting of one or two β-hydroxy fatty acids. Different rhamnolipid congeners can be found, depending on the length of the fatty acid units (8-16 carbon atoms) and the degree of saturation. rhamnolipids are usually produced as mixtures of different congeners, and to date more than 60 different rhamnolipids have been identified (Abdel-Mawgoud et al., 2010; Funston et al., 2016; HoŠková et al., 2013). Besides their excellent surface active properties, rhamnolipids have been reported to exhibit interesting biological activities, including antifungal activity (Rodrigues et al., 2017). However, their effect on the production of mycotoxins has not been reported yet.

The aim of this chapter was to study the effect of rhamnolipids on the growth and aflatoxins production by *A. flavus* MUM 17.14. Two different rhamnolipids mixtures, obtained from *Pseudomonas aeruginosa* #112 and *Burkholderia thailandensis* E264, were

evaluated. Furthermore, the individual effect of mono- and di-rhamnolipids produced by *P. aeruginosa* #112 was also studied.

4.2. MATERIALS AND METHODS

4.2.1. Microorganisms and culture conditions

The bacterial strains *P. aeruginosa* #112 and *B. thailandensis* E264 (DSM 13276) were used for the production of rhamnolipids. *P. aeruginosa* #112 was isolated from a crude oil sample obtained from a Brazilian oil field (Gudiña et al., 2015). *B. thailandensis* E264 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The bacterial strains were stored at -80ºC in Luria Bertani (LB) medium (for *P. aeruginosa*) or nutrient broth (NB) (for *B. thailandensis*) supplemented with glycerol (20%, v/v). The composition of LB medium was (g/L): NaCl 10; tryptone 10; yeast extract 5; pH 7. The composition of NB was (g/L): peptone 5; meat extract 3; pH 7.

The mycotoxigenic fungi *A. flavus* MUM 17.14, which produced AFB_1 (together with very low levels of AFB_2), was obtained from the culture collection of Micoteca da Universidade do Minho (MUM), Portugal. It was maintained at -80ºC in sterile demineralized water supplemented with glycerol (20%, v/v). Whenever required, frozen stocks were streaked on Malt Extract Agar (MEA, Blakeslee's formula) plates and incubated at 25ºC for 7 days. Subsequently, the agar plates were stored at 4ºC no longer than 3 weeks. The composition of MEA medium was (g/L): malt extract 20; glucose 20; peptone 1; agar 20; pH 5.5.

4.2.2. Agro-industrial residues

Corn steep liquor (CSL) and molasses were kindly provided by COPAM: Companhia Portuguesa de Amidos, S.A. (Portugal) and RAR: Refinarias de Açúcar Reunidas, S.A. (Portugal), respectively. The composition of these residues was reported in Chapter 2.

4.2.3. Rhamnolipids production, recovery and purification

The production, recovery and separation of rhamnolipids by *P. aeruginosa* #112 was performed as describe in previous chapters. Subsequently, the mono- and di-rhamnolipid

congeners present in the crude rhamnolipid mixture were separated and purified through silica gel column chromatography as described Chapter 3.

Rhamnolipids production by *B. thailandensis* E264 was performed in NB supplemented with glycerol (4%, v/v). The assays were performed in 500 mL Erlenmeyer flasks containing 200 mL of culture medium. Each flask was inoculated with 1% (v/v) of a pre-culture grown in the same culture medium at 30ºC and 180 rpm for 24 h. The flasks were incubated at the same conditions up to 60 h. At the end of the fermentation, the cultures were centrifuged (10 000 × g, 20 min, 20°C) and the rhamnolipids produced were extracted from the cell-free supernatants with ethyl acetate, as described by Funston and co-workers (2016).

All the products obtained were freeze-dried, weighed and stored at -20ºC for further experiments. Along the production, recovery and purification of rhamnolipids, the surface tension and critical micelle concentration (*cmc*) was measured as described in Chapter 2, and the presence of rhamnolipids was verified through thin layer chromatography according to Chapter 3.

4.2.4. Effect of rhamnolipids on aflatoxins production

The antifungal activity of the rhamnolipids produced by *P. aeruginosa* #112 and *B. thailandensis* E264 against *A. flavus* MUM 17.14 was evaluated. The assays were performed in Petri dishes (55 mm diameter) containing MEA medium supplemented with the freeze-dried rhamnolipids (crude rhamnolipid mixtures and purified mono- and di-rhamnolipids congeners) at different concentrations (45-1500 mg/L). The agar plates were inoculated with 10 µL of a spore suspension of *A. flavus* MUM 17.14 (prepared in sterile demineralized water at a concentration of 10^5 spores/mL, according to Chapter 3 in the center of the plate. Subsequently, the plates were incubated at 25ºC for 5 days. Control assays were performed using MEA medium without rhamnolipids. The fungal growth was determined by measuring the diameter of the growth zone. The percentage of growth inhibition relative to the control was calculated as described in Chapter 3. The effect of the rhamnolipids produced by *P. aeruginosa* #112 and *B. thailandensis* E264 on the production of aflatoxins by *A. flavus* MUM 17.14 was studied using the same plates at the end of the incubation period (5 days). The entire content of each plate was cut into pieces, transferred to a 50 mL tube and extracted with 20 mL of a mixture containing

acetonitrile:methanol:acetic acid (78:20:2, v/v/v) by agitation with vortex at high speed. The extracts were left overnight at room temperature in the dark. Subsequently, 2 mL of each extraction were filtered through a 0.2 µm syringe filter and analyzed by HPLC with fluorescence detection according to the methodology described by Guimarães et al. (2018). The detection (S/N of 2:1) and quantification limit (S/N of 10:1) of the method were 3.3 and 16.5 ng/mL, respectively. Repeatability was evaluated by calculating a pooled RSD using 24 samples performed in triplicate (RSD_{pooled}= 19%). Mean recoveries (98 ± 9%) were evaluated by extracting plates containing MEA medium supplemented with 3 µg of AFB_1 in triplicate. The HPLC system used was equipped with a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a photochemical post-column derivatization reactor (PHRED unit, Aura Industries, USA) and a Jasco FP-920 fluorescence detector. The instrument and the chromatographic data were managed by a Varian 850-MIB data system interface and a Galaxie chromatography data system, respectively. A C_{18} reversed-phase YMC-Pack ODS-A analytical column (250 × 4.6 mm i.d., 5 µm particle size) connected to a guard column with the same stationary phase was used. The mobile phase used was a mixture of water:acetonitrile:methanol (3:1:1, v/v/v), and the column oven temperature was set to 30ºC. The injection volume was 30 µL, and the compounds were eluted at a flow rate of 1.0 mL/min for a 20 min isocratic run. The fluorescence of aflatoxins was recorded at excitation and emission wavelengths of 365 and 435 nm, respectively. Calibration curves of aflatoxins were prepared in mobile phase using a certified aflatoxins standard solution mixture (46304-U, Sigma-Aldrich, USA) at a concentration range of 50-1000 ng/mL. Aflatoxins were quantified by comparing the peak areas in the samples with those of the calibration curves. All the analyses were performed in triplicate.

4.2.5. Statistical analysis

All data were expressed as the means ± standard deviation of three independent replicates. Results were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's HSD multiple comparisons test. All statistical analyses were performed at a significance level of 0.05 using the software IBM SPSS Statistics 22 (IBM Corp., Armonk, USA).

4.3. RESULTS AND DISCUSSION

4.3.1. Antifungal activity of rhamnolipids against *A. flavus* MUM 17.14

The development of new environmentally-friendly treatments to reduce and/or eliminate mycotoxins in food is of utmost importance, hence in this work the effect of rhamnolipids on the growth of *A. flavus* MUM 17.14, as well as on the production of aflatoxins, was evaluated.

P. aeruginosa #112 was previously reported to produce eight different rhamnolipid congeners, including mono- and di-rhamnolipids with acyl chains containing 8, 10 or 12 carbons. Among them, the most abundant congeners were the mono-rhamnolipid Rha-C_{10}-C_{10} and the di-rhamnolipid Rha-Rha-C_{10}-C_{10} (45% and 26% of the total rhamnolipids, respectively) (Gudiña et al., 2015). On the other hand, the rhamnolipids produced by *B. thailandensis* E264 contain longer fatty acid moieties (12, 14 and 16 carbons). This strain produces mainly di-rhamnolipids (93% of the total rhamnolipids), being the most abundant congener Rha-Rha-C_{14}-C_{14} (77%) (Dubeau et al., 2009).

Due to the different distribution of congeners, the rhamnolipids produced by *P. aeruginosa* #112 and *B. thailandensis* E264 exhibit different properties. The minimum surface tension value achieved with the rhamnolipids produced by *P. aeruginosa* #112 was 29.9 ± 0.5 mN/m, whereas in the case of *B. thailandensis* E264 this value was 32.2 ± 1.3 mN/m. Furthermore, rhamnolipids from *P. aeruginosa* #112 exhibited a lower *cmc* (50 mg/L) when compared with those from *B. thailandensis* E264 (250 mg/L). These values are in accordance with those previously reported in the literature for both microorganisms (Dubeau et al., 2009; Gudiña, et al., 2015).

The antagonistic activity of rhamnolipids against *A. flavus* MUM 17.14 was first evaluated using the crude rhamnolipid mixtures produced by both microorganisms at different concentrations. As it can be seen in Table 4.1, the rhamnolipids produced by *P. aeruginosa* #112 exhibited a higher inhibitory activity comparing to the ones from *B. thailandensis* E264 (statistically significant differences ($p < 0.05$) for all concentrations tested), which can be due to the presence of different rhamnolipid congeners. In the case of *P. aeruginosa* #112, the growth inhibition percentages obtained for crude rhamnolipid concentrations between 90 and 1500 mg/L (34 to 40%) were not significantly different.

These results are in accordance with previous studies that reported antifungal activity of rhamnolipids produced by *P. aeruginosa* against other genera of fungi, including *Botrytis*, *Colletotrichum*, *Fusarium* and *Phytophthora*, at concentrations between 30 and 450 mg/L (Kim et al., 2000; Reddy et al. 2016; Sha et al., 2012). The inhibitory activity of the crude rhamnolipid mixture produced by *P. aeruginosa* #112 can be seen in Figure 4.1.

Table 4.1. Growth inhibition percentages obtained for *Aspergillus flavus* MUM 17.14 with the crude rhamnolipid mixtures produced by *Burkholderia thailandensis* E264 (RL_{Bt}) and *Pseudomonas aeruginosa* #112 (RL_{Pa}), and with the purified mono- and di-rhamnolipids (from *P. aeruginosa* #112) at different concentrations. The assays were performed at 25°C for 5 days. The results represent the average of three independent experiments ± standard deviation.

	Growth Inhibition (%)			
[RL] mg/L	**Crude RL_{Bt}**	**Crude RL_{Pa}**	**Mono-RL**	**Di-RL**
0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
45	4.0 ± 3.4^{aC}	23.5 ± 2.8^{bB}	27.5 ± 1.5^{bB}	37.8 ± 0.0^{bA}
90	2.7 ± 2.3^{aB}	38.9 ± 1.8^{cA}	36.8 ± 3.1^{cA}	40.9 ± 2.3^{bA}
190	4.0 ± 1.9^{aC}	40.9 ± 1.8^{cB}	43.5 ± 2.3^{dAB}	47.2 ± 1.0^{cA}
375	4.0 ± 0.1^{aC}	34.7 ± 2.0^{cB}	47.2 ± 1.0^{dA}	46.1 ± 2.5^{cA}
750	3.1 ± 1.7^{aB}	39.5 ± 6.2^{cA}	46.6 ± 0.9^{dA}	46.1 ± 2.5^{cA}
1500	6.7 ± 0.0^{bC}	40.1 ± 2.4^{cB}	36.1 ± 4.5^{cB}	49.5 ± 2.7^{cA}

Different lower-case letters within the same column and different capital letters within the same row indicate statistically significant differences ($p < 0.05$).

Although the antimicrobial activity of rhamnolipids produced by different *P. aeruginosa* strains has been extensively studied, to the best of our knowledge, the antimicrobial activity of rhamnolipids produced by *B. thailandensis* has only been reported by Elshikh and co-workers.(2017a). These authors concluded that long-chain rhamnolipids (Rha-Rha-C_{14}-C_{14}) exhibited a similar inhibitory activity against oral pathogens (*Streptococcus sanguinis*, *Streptococcus oralis*, *Actinomyces naeslundii* and *Weisseria mucosa*) as compared to short

chain rhamnolipids (Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10}) (Elshikh et al., 2017a; 2017b). On the contrary, the results herein presented indicate a higher inhibitory activity of short chain rhamnolipids against *A. flavus* MUM 17.14 when compared with long-chain rhamnolipids (Table 4.1).

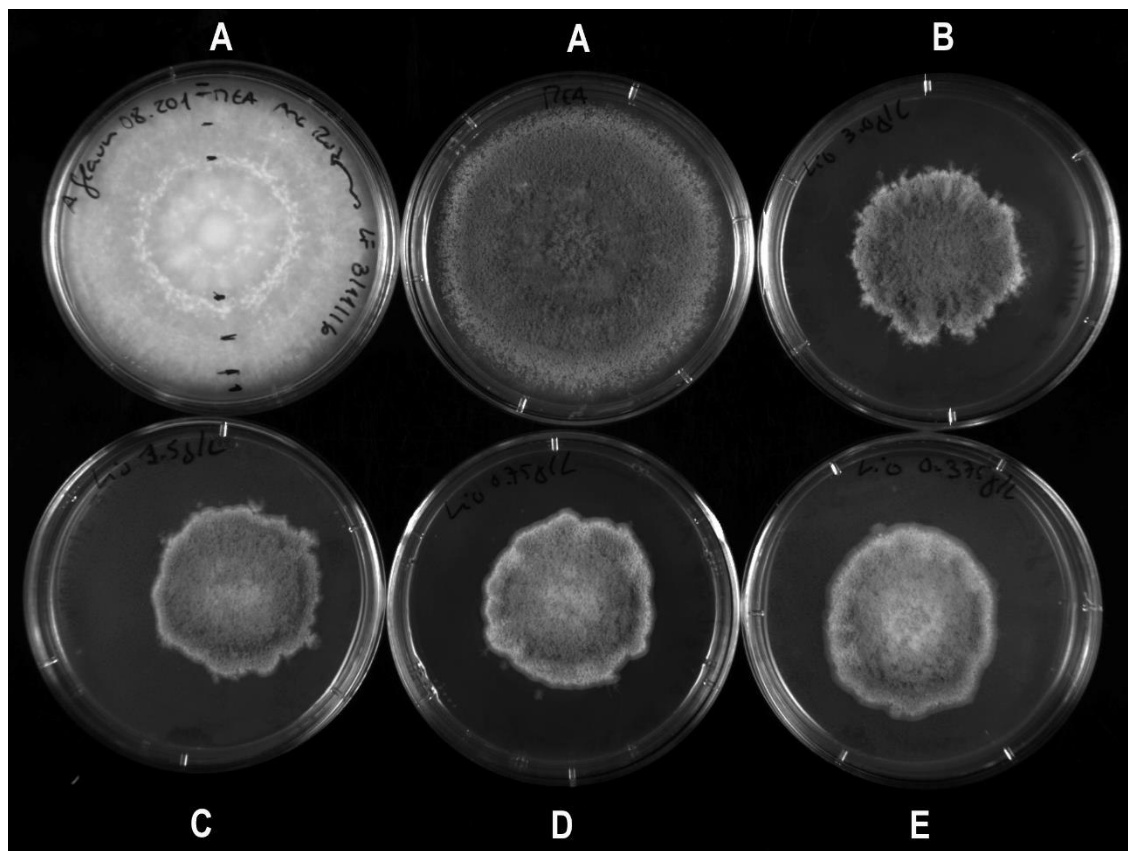

Figure 4.1. Antifungal activity of the crude rhamnolipid mixture produced by *Pseudomonas aeruginosa* #112 at different concentrations against *Aspergillus flavus* MUM 17.14. A: 0 mg_{RL}/L; B: 3000 mg_{RL}/L; C: 1500 mg_{RL}/L; D: 750 mg_{RL}/L; E: 375 mg_{RL}/L.

Subsequently, the individual contribution of mono- and di-rhamnolipids to the antifungal activity observed was assessed. The purified mono- and di-rhamnolipids dissolved in demineralized water reduced the surface tension up to 25.9 ± 0.2 mN/m (*cmc* 50 mg/L) and 33.5 ± 0.3 mN/m (*cmc* 15 mg/L), respectively. However, as seen from the results in Table 4.1, despite their different surface activity, both congeners exhibited a similar inhibitory activity against *A. flavus* MUM 17.14. Only at the lowest and the highest concentrations tested, di-rhamnolipids exhibited a statistically significant higher antagonistic activity ($p < 0.05$) comparing to mono-rhamnolipids.

Contrary to the results herein reported, Rodrigues and co-workers (2017) previously demonstrated that di-rhamnolipids exhibited a remarkable inhibitory activity against mycotoxin-producing *Aspergillus niger* and *Aspergillus carbonarius* strains, whereas mono-rhamnolipids showed a weak inhibitory activity. The same authors also demonstrated that sodium chloride considerably increased the antifungal activity of rhamnolipids against *A. niger* and *A. carbonarius* strains due to a change in their aggregation behavior (Rodrigues et al., 2017). For that reason, the antifungal activity of the crude rhamnolipids produced by *P. aeruginosa* #112 against *A. flavus* MUM 17.14 was also studied at different NaCl concentrations (500-1000 mM). However, in this case, an increase in the antifungal activity was not observed (*data not shown*).

The mechanisms involved in the antifungal activity of rhamnolipids are not well established, and it is usually assumed that due to their detergent-like effect they interact with the biological membranes, disturbing their integrity and permeability by forming pores and ion channels which ultimately results in cell lysis (Mnif et al., 2016; Sánchez et al., 2007). However, according to the results herein presented, it can be concluded that the antifungal activity of rhamnolipids and the effect of the different congeners is dependent on the fungal species, which suggests a more specific mode of action (*e.g.* modifying the gene expression profile in the target microorganism; protein denaturation (Andersen and Otzen, 2014; Rodriguez and Mahoney, 1994). It can also be hypothesized that the differences observed in the inhibitory activity exhibited by the crude rhamnolipid mixtures produced by *P. aeruginosa* #112 and *B. thailandensis* E264 can be due to the different lengths of the fatty acid chains of rhamnolipids and not to the proportion of mono- and di-rhamnolipids present in the mixture, as both congeners exhibit a similar activity.

Lipopeptide biosurfactants produced by *Bacillus* species have been reported to exhibit a strong antifungal activity against aflatoxin-producing *A. flavus* strains. Among the different lipopeptide families (iturins, fengycins and surfactins), iturin A and bacillomycin D (both of them belonging to the iturin family) demonstrated to be the most effective, although they exhibited lower surface activity when compared to surfactin (Afsharmanesh et al., 2014; Gong et al., 2014; Kimura and Hirano, 1988). In the case of bacillomycin D, growth inhibition achieved 85% at a concentration of 200 mg/L (Gong et al., 2014). Several studies demonstrated that these biosurfactants alter the integrity of the fungal cell wall and the permeability of the cytoplasmic membrane (Afsharmanesh et al., 2014; Gong et al., 2014). According to Moyne and co-workers (2001), ergosterol and cholesterol could be the target

of bacillomycin D, and the length of the fatty acid chain of bacillomycin D (15 or 16 carbons) plays an important role in the antifungal activity of this lipopeptide biosurfactant. However, in the case of *A. flavus*, both bacillomycin analogues exhibited the same activity (Moyne et al., 2001). Ergosterol was also identified as target for the antifungal compounds (probably lipopeptide biosurfactants) present in the cell-free supernatants of cultures of *Bacillus amyloliquefaciens* UTB2 and *B. subtilis* UTB3, which reduced the growth of the aflatoxigenic fungus *Aspergillus parasiticus* NRRL 2999 by 90% (Siahmoshteh et al., 2018).

4.3.2. Effect of rhamnolipids on aflatoxins production

The effect of rhamnolipids on the production of aflatoxins by *A. flavus* MUM 17.14 was studied using the crude rhamnolipid mixtures produced by *P. aeruginosa* #112 and *B. thailandensis* E264 at different concentrations. Analyzing Table 4.2, it can be seen that at concentrations higher than 90 mg/L, no statistically significant differences ($p < 0.05$) were observed between the crude rhamnolipids produced by both microorganisms at the same concentration. At the highest concentration tested (1500 mg/L), the crude rhamnolipids produced by *B. thailandensis* and *P. aeruginosa* inhibited the production of AFB_1 by 99.8% and 99.5%, respectively. However, at lower concentrations (45 and 90 mg/L), a statistically significant higher inhibition ($p < 0.05$) was achieved with the crude rhamnolipids produced by *P. aeruginosa* #112 (around 94%) comparing to those from *B. thailandensis* E264 (56-65%). Regarding the effect of the mono- and di-rhamnolipid congeners, at concentrations between 190 and 1500 mg/L, di-rhamnolipids exhibited a statistically significant higher inhibition ($p < 0.05$) than the mono-rhamnolipids. At a concentration of 1500 mg/L, both congeners inhibited AFB_1 production by 98-99%.

Table 4.2. Aflatoxin B_1 (AFB_1) concentration and percentage of AFB_1 reduction (relative to the control) in cultures of *Aspergillus flavus* MUM 17.14 grown in the presence of crude rhamnolipid mixtures produced by *Burkholderia thailandensis* E264 (RL_{Bt}) and *Pseudomonas aeruginosa* #112 (RL_{Pa}), and in the presence of purified mono- and di-rhamnolipids (from *P. aeruginosa* #112) at different concentrations. The results represent the average of three independent experiments ± standard deviation.

	AFB_1 concentration (ng/mL) (AFB_1 reduction (%))			
[RL] mg/L	**Crude RL_{Bt}**	**Crude RL_{Pa}**	**Mono-RL**	**Di-RL**
0	3393 ± 122^{a}	3393 ± 122^{a}	3393 ± 122^{a}	3393 ± 122^{a}
45	1495 ± 77^{bAB} (55.9 ± 2.2)	205 ± 19^{bC} (93.9 ± 0.5)	1297 ± 130^{bcB} (61.8 ± 0.4)	1850 ± 268^{bA} (45.5 ± 7.9)
90	1173 ± 258^{bB} (65.4 ± 7.6)	218 ± 23^{bC} (93.6 ± 0.6)	1372 ± 189^{bAB} (59.6 ± 6.6)	1658 ± 74^{bA} (51.1 ± 2.2)
190	407 ± 96^{cBC} (88.1 ± 2.8)	212 ± 56^{bB} (93.7 ± 1.6)	1013 ± 132^{cdA} (70.1 ± 3.9)	565 ± 86^{cC} (83.3 ± 2.5)
375	103 ± 7^{cdB} (96.9 ± 0.2)	74 ± 21^{bcB} (97.8 ± 0.6)	816 ± 160^{deA} (75.9 ± 4.7)	116 ± 50^{dB} (96.6 ± 1.4)
750	111 ± 28^{cdB} (96.7 ± 0.8)	43 ± 18^{cB} (98.7 ± 0.5)	559 ± 65^{eA} (83.5 ± 1.9)	58 ± 1^{dB} (98.3 ± 0.0)
1500	7 ± 2^{dB} (99.8 ± 0.0)	16 ± 1^{cB} (99.5 ± 0.0)	72 ± 2^{fA} (97.9 ± 0.0)	22 ± 2^{dB} (99.3 ± 0.0)

Different lower-case letters within the same column and different capital letters within the same row indicate statistically significant differences ($p < 0.05$).

The high inhibitions observed for the crude rhamnolipids produced by *P. aeruginosa* #112 at concentrations of 45 and 90 mg/L can only be the result of a synergistic effect due to the combination of mono- and di-rhamnolipids. In the case of *B. thailandensis* E264, as it produced mainly di-rhamnolipids, this synergistic effect was not observed and, consequently, the inhibitions obtained at those concentrations were significantly lower ($p < 0.05$). Similar synergistic effects were previously reported for lipopeptide biosurfactants. Surfactin, despite its outstanding surface activity, exhibits a weak antifungal activity. However, it considerably increases the antifungal activity of iturin A and fengycins (Pereyra et al., 2018).

Another possible explanation for the lower activity of rhamnolipids from *B. thailandensis* E264 at low concentrations as compared to those from *P. aeruginosa* #112 is the difference in the length of the fatty acid chains of both rhamnolipids. Rodriguez and Mahoney (1994) reported an inverse correlation between aflatoxin inhibition by different synthetic surfactants and the length of their hydrophobic chains. In some cases, an increase in the hydrophobic chain length from 12 to 18 carbons reduced the inhibitory effect by 50%. However, it is important to highlight that although the rhamnolipids produced by *B. thailandensis* E264 did not have a significant effect on fungal growth, at high concentrations they exhibited a similar inhibition of the AFB_1 production comparing to the crude rhamnolipids from *P. aeruginosa* #112.

Similarly, to the results herein obtained, previous works demonstrated an inhibition of aflatoxins production not directly related to the inhibition of fungal growth. The cell-free supernatants of cultures of *Lactobacillus plantarum* UM55 inhibited the growth of *A. flavus* MUM 17.14 (the same strain used in this study) by 32%, whereas the production of aflatoxins was reduced by 95%. This inhibitory effect was due to the presence of different organic acids, being phenyllactic acid the most relevant (Guimarães et al., 2018) .

The effect of lipopeptide biosurfactants on aflatoxin production has been previously reported by other authors. The production of AFB_1 by *A. flavus* A12 was reduced by 99.8% when it was grown in co-culture with two different *Bacillus* sp. strains due to the production of the lipopeptide biosurfactants iturin A and surfactin (Veras et al., 2016). Likewise, when *A. parasiticus* NRRL 2999 was grown in co-culture with *Bacillus mojavensis* RC1A (which produced the lipopeptide biosurfactants surfactin, iturin A and fengycin), the AFB_1 production was reduced by 97.5% (Pereyra et al., 2018). Iturin A produced by *B. subtilis*

NK-330 completely inhibited the production of aflatoxins by *A. parasiticus* NRRL 2999 and *A. flavus* NRRL 3357 (Kimura and Hirano, 1988).

The mechanism of action of biosurfactants on the production of aflatoxins is still not fully understood. Rodriguez and Mahoney (1994) hypothesized that surfactants can act on aflatoxin-biosynthetic enzymes located in the membrane of the fungi, causing their denaturation. The rhamnolipids effect on the production of aflatoxins holds a great promise regarding their potential applications, therefore further studies ought to be performed to unravel their mechanisms of action.

As natural products synthesized by microorganisms, rhamnolipids have been extensively studied for application in cosmetics and pharmaceutics, as well as in agriculture and in the food industry, due to their low toxicity and high biodegradability (Kim et al., 2000; Reddy et al., 2016; Rodrigues et al., 2017; Sha et al., 2012). Rhamnolipids have been proved to improve the texture, volume, consistency, dough stability and conservation of baked products, without altering their sensorial properties (Haesendonck and Vanzeveren, 2006). The applicability of rhamnolipids in agriculture, as an alternative to the chemical surfactants, to improve the foliar uptake of herbicides by modifying the wetting leaf surface and enhancing penetration across the cuticular membrane, was also demonstrated (Liu et al., 2016). Furthermore, the commercial biofungicide Zonix™ (NOP Supply LLC., USA), which includes rhamnolipids in its formulation, has been approved by the Food and Drug Administration (FDA) to be directly used on vegetables, legumes, and fruits crops to prevent contamination by pathogenic fungi, due to its low mammalian toxicity and non-mutagenicity (https://www3.epa.gov/pesticides/chem_search/ppls/072431-00001-20040323.pdf).

4.4. CONCLUSIONS

In this work, the inhibitory effect of rhamnolipids on the growth and aflatoxins production by *A. flavus* MUM 17.14 was reported for the first time. It was demonstrated that the effect of rhamnolipids on the growth and aflatoxins production is different for the mono- and di-rhamnolipid congeners, and in some cases, a synergistic effect was observed due to the combination of different congeners. Although none of the rhamnolipids herein studied inhibited the growth of *A. flavus* MUM 17.14 more than 40%, surprisingly all of them almost completely inhibited the production of aflatoxins. Consequently, these

compounds can be considered a promising environmentally-friendly alternative to reduce and/or eliminate the presence of aflatoxins in food.

4.5. REFERENCES

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CHAPTER 5

EFFECT OF RHAMNOLIPIDS IN ASEXUAL DEVELOPMENT AND AFLTOXINS BIOSYNTHESIS

ABSTRACT

Aflatoxins are naturally carcinogenic compounds that can be found in different foods and feed. Aflatoxin concentration in these products need to be reduced as much as possible. One of the strategies to achieve that could be by using "green" compounds, such as the rhamnolipids that exhibit antifungal activity against different mycotoxigenic fungi including *Aspergillus flavus*. In this study, we investigated the rhamnolipids inhibitory effect on the asexual development and aflatoxin production in *A. flavus* MUM 17.14. Rhamnolipids were found to inhibit the expression of the genes *afl*C and *afl*Q, which are involved in the aflatoxins biosynthesis. The reduction of the expression of these genes could explain the complete inhibition of aflatoxin production by *A. flavus* MUM 17.14 when exposed to rhamnolipids. Transmission electron microscopy analysis showed that rhamnolipids cause several damages in the internal hyphae content. However, this could not be confirmed from a genetic point of view since, apparently the exposure to rhamnolipids does not affect the expression of the genes *aba*A and *wet*A that are involved in the asexual development. Further studies are required to unravel the mechanisms underlying the effect of rhamnolipids effect in the asexual development and aflatoxin biosynthesis. Overall, our results strongly suggest that rhamnolipids are a promising alternative to use as an anti-aflatoxin agent.

Keywords: Aflatoxins biosynthesis, Rhamnolipids, Asexual development, Hyphae, Spores

CONTENTS

5.1. INTRODUCTION........126

5.2. MATERIAL AND METHODS........128

5.2.1. MICROORGANISMS AND CULTURE CONDITIONS........128

5.2.2. RHAMNOLIPIDS PRODUCTION AND RECOVERY........128

5.2.3. CONIDIAL PRODUCTION ANALYSIS........128

5.2.4. ANTI-MYCOTOXIN ASSAYS........129

5.2.4.1. ISOLATION OF TOTAL RNA AND RT-PCR........129

5.2.4.2. AFLATOXINS ANALYSIS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)........132

5.2.5. TRANSMISSION ELECTRON MICROSCOPY (TEM)........132

5.2.6. STATISTICAL ANALYSIS........132

5.3. RESULTS AND DISCUSSION........133

5.3.1. CONIDIAL PRODUCTION AND ANALYSIS OF THE COLONY MORPHOLOGY BY TEM........133

5.3.2. RT-PCR ANALYSIS OF THE EXPRESSION OF GENES INVOLVED IN SPORULATION........135

5.3.3. RT-PCR ANALYSIS OF THE EXPRESSION OF GENES INVOLVED IN AFLATOXIN BIOSYNTHESIS…… 137

5.4. CONCLUSIONS........140

5.5. REFERENCES........140

5.1. INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by different filamentous fungi known for contaminating several food and feed crops causing serious risks for human and animal health and substantial economic losses (Barrett, 2000; Pankaj et al., 2018). Approximately 400 compounds are recognized as mycotoxins, but the most commonly observed mycotoxins that present a concern to human and animal health include aflatoxins, ochratoxin A, patulin, fumonisins, and zearalenone (Bennett and Klich, 2003). Among them, aflatoxin is one of the mycotoxins representing a major concern in agriculture (Mwakinyali et al., 2019). Aflatoxins were first discovered in 1960 as the result of an epidemic of "Turkey X disease" were more than 100 000 turkeys died in England after the consumption of mold-contaminated peanut meal (Barrett, 2000; Rushing and Selim, 2019). Aflatoxins are produced by *Aspergillus* species, mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi widely contaminate different types of crops in tropical and sub-tropical regions, such as maize, peanuts, wheat, barley, and rice (Deng et al., 2018; Guimarães et al., 2018; Rushing and Selim, 2019). Approximately 20 aflatoxins have been identified and 4 of them occur naturally, including aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) (Deng et al., 2018). *A. flavus* is known to produce the two type of aflatoxins AFB_1 and AFB_2 (Yu et al., 2004). In fact, AFB_1 has been classified as Group I carcinogen by International Agency for Research on Cancer (IARC, 2002).

Reducing aflatoxins exposure in many agriculture products is extremely important. Hence, it is essential to find a good "green" control method to replace the hazardous chemicals fungicides that are not only harmful for human health and environment, but also lead to the development of new resistant strains (Guimarães et al., 2018; Pekmezovic et al., 2015). Biosurfactants are eco-friendly compounds that could potentially be used for this purpose as biocontrol agents. For instance, rhamnolipids have been found, as reported in the previous chapters, to have an antagonistic effect against *Aspergillus* species, namely *Aspergillus niger* (Rodrigues et al., 2017), *Aspergillus carbonarius* (Rodrigues et al., 2017) and *A. flavus* (Chapter 4), which are well-known to produce mycotoxins. Other authors reported the rhamnolipids antifungal activity against other mycotoxigenic fungi, as for example, *Fusarium oxysporium* (Reddy et al., 2016) and *Fusarium verticillioides* (Borah et al., 2016).

In order to develop a novel and effective strategy against *A. flavus* contamination, not only inhibiting its growth, but also decreasing the aflatoxin production, it is of utmost relevance to understand the molecular mechanisms by which asexual sporulation (conidiation) and aflatoxin biosynthesis are regulated. Conidiation in filamentous fungi involves the formation of conidia, formed on specialized structures called conidiophores. Conidiation is regulated by multiple positive and negative genetic elements that direct the expression of the genes required for a suitable vegetative growth and the assembly of the conidiophore and spore maturation (Park and Yu, 2012). The genetic mechanisms of conidiation have been extensively studied in the *Aspergillus* species, mainly in the fungal *Aspergillus nidulans* (Lee et al., 2016). The genes *brl*A (bristle), *aba*A (abacus), and *wet*A (wet-white conidia) are expressed sequentially and form the central developmental pathway of conidiation formation. The *brl*A gene is a transcription factor that mediates the budding growth of conidiophores (Chang et al., 2012). The *aba*A gene controls phialide differentiation and is activated by the *brl*A gene during the middle stages of conidiophores (Lee et al., 2016; Park and Yu, 2012). Subsequently, *aba*A gene mediates transcriptional activation of *wet*A in late phase of conidiation for synthesis of crucial cell-wall components and conidial metabolic remodeling, that in turn activates the expression of *brl*A (Chang et al., 2012; Lee et al., 2016). The *wet*A gene is proposed to activate a set of genes with spore specific functions (Park and Yu, 2012). Alterations in the above mentioned genes expression could result in abnormal proliferation of undifferentiated vegetative hyphae (Chang et al., 2012; Park and Yu, 2012).

Aflatoxins are polyketide derived secondary metabolites produced via the following conserved pathway: in the first stage an acetate molecule is converted into a polyketide molecule; then in a second stage the polyketide molecule is converted into anthraquinones molecules that are afterwards transformed into xanthones molecules; and in the last stage, the xanthones molecules are converted into the final aflatoxins forms (Yu et al., 2004). Aflatoxin biosynthesis has been proposed to involve at least 23 enzymatic reactions and more than 30 genes or open reading frames (ORFs) representing a well-defined aflatoxin pathway gene cluster (Kong et al., 2010; Wang et al., 2017; Yu et al., 2004).

In the previous chapter it was demonstrated that the rhamnolipids produced by *Pseudomonas aeruginosa* #112 were able to reduce the *A. flavus* MUM 17.14 growth and, at the highest concentrations tested, to completely inhibit the aflatoxin production. Therefore, the aim of this chapter was to evaluate the rhamnolipids effect in the regulation

of different genes involved in the aflatoxins biosynthesis and sporulation by *A. flavus* MUM 17.14. Furthermore, given the morphological alterations observed in *A. flavus* MUM 17.14 when exposed to rhamnolipids, that resulted from its antifungal activity, the intracellular structures were evaluated through electron microscopy analysis.

5.2. MATERIAL AND METHODS

5.2.1. Microorganisms and culture conditions

The bacterial strain *P. aeruginosa* #112 was used for rhamnolipids production. It was stored at -80ºC in Luria Bertani (LB) medium supplemented with 20% (v/v) of glycerol. The composition of LB medium was (g/L): NaCl 10.0; tryptone 10.0; yeast extract 5.0; pH 7.0. The fungal strain *A. flavus* MUM 17.14 was obtained from the culture collection of Micoteca of Universidade do Minho (MUM, Portugal). It was maintained at -80ºC in sterile demineralized water supplemented with 20% (v/v) of glycerol. Whenever required, frozen stocks of the fungi were streaked on Malt Extract Agar (MEA) plates and incubated at 25ºC for 7 days. The agar plates were stored at 4ºC no longer than 3 weeks. The composition of MEA medium (Blakeslee's formula) was (g/L): malt extract 20.0; glucose 20.0; peptone 1.0; agar 20.0; pH 5.5.

5.2.2. Rhamnolipids production and recovery

The production of rhamnolipids by *P. aeruginosa* #112 was performed in 1000 mL flasks containing 400 mL of culture medium Corn Steep Liquor (CSL) 10% (v/v) and molasses 10% (w/v) (CSLM), as previously described in Chapter 3. The rhamnolipids produced were recovered from cell-free supernatants by adsorption chromatography using the polystyrene resin Amberlite XAD-2 as previously described in Chapter 2. The rhamnolipids mixture was quantified as the dry weight of the freeze-dried product.

5.2.3. Conidial production analysis

To analyze the effect of the rhamnolipids in the *A. flavus* MUM 17.14 conidial production, an aliquot with 10^5 spores/mL was spread onto 5 mL of MEA agar medium in a 15 mL falcon with and without the presence of rhamnolipids. The concentration of rhamnolipids used for these assays was 1.5 $g_{rhamnolipids}$/mL. The different cultures were

incubated in the dark at 25ºC for 5 days. At the end of the growth period, the conidial was harvested by washing it out from the agar through the addition of 1 mL of 0.01% Tween 20 solution until the last volume of 10 mL. The conidial solution was counted using a Neubauer improved cell counter (Marienfeld GmbH, Germany). For a quantitative comparison of the conidial production the assays were made in triplicate.

5.2.4. Anti-mycotoxin assays

A. flavus MUM 17.14 spores at concentration of 10^6 were inoculated into 50 mL of MEA liquid medium with two different conditions, with and without the addition of rhamnolipids produced by the strain *P. aeruginosa* #112. The rhamnolipids concentration used was 1.5 g/L and the culture medium without rhamnolipids was used as control. The cultures were incubated at 28ºC at 150 rpm for 3 days. Four replicates of each condition were performed. At the end of the fermentation, the culture medium was centrifuged at 9000 rpm for 20 min. The supernatant was decanted to completely remove the liquid from the fungal mycelial. The fungal mycelial was immediately frozen at -80ºC for further extraction of the RNA and the supernatant was maintained at -20ºC and used to evaluate the aflatoxins production by HPLC.

5.2.4.1. Isolation of total RNA and RT-PCR

A. flavus MUM 17.14 mycelia recovered from the liquid cultures was placed in a mortar and ground to a fine powder with an appropriated amount of liquid nitrogen. Total RNA was extracted from the *A. flavus* MUM 17.14 mycelia according to the protocol from Plant/Fungi Total RNA Purification Kit (NORGEN, Biotek Corp). Extracted RNA was quantified by determining the absorbance at 260 and 280 nm using a Nanodrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.). The RNA quality was evaluated using agarose gel (0.8%, w/v) electrophoresis. The RNA samples were treated with DNase I, RNase-free from Thermo Fisher Scientific according to the respective protocol. The complementary DNA (cDNA) was prepared using GrispXpert cDNA Synthesis Mastermix Kit (Grisp Research Solution). A Xpert Fast SYBR kit (Grisp Research Solution) was used for RT-PCR analysis, according with the protocol specifications. Specific primers synthesized by Metabion International AG and Eurofins Genomics were used to analyze the rhamnolipids effect on aflatoxin biosynthesis and

sporulation. The primers for the genes involved in aflatoxins biosynthesis (*afl*R, *afl*S, *afl*C, *afl*Q and β-tubulin) and sporulation (*aba*A, *brl*A and *wet*A) are listed in the Table 5.1.

Table 5.1. Gene-specific primers from aflatoxins biosynthesis and sporulation used for RT-PCR. [*E*]: primers efficiency. [Tm]: primer melting temperature.

Target gene	Sequence (5'-->3')	*E*	Tm (^{0}C)	Reference
*afl*R	F- GGGAACAAGAGGGCTACCGA	2.06	61.26	Kong, et al., 2010
	R - TGCCAGCACCTTGAGAACG		60.60	
*afl*S	F - GGTCGTGCATGTGCGAATC	2.01	59.94	Kong et al., 2010
	R - GAGGGCAACAACCAGTGAGG		60.89	
*afl*C	F - TGCATGGCGATGTGGTAGTT	2.06	60.04	Moon et al., 2018
	R - GTAAGGCCGCGAGAGAAAG		58.33	
*afl*Q	F - GTCGCATATGCCCCGGTCGG	2.02	65.72	Wang et al., 2017
	R - GGCAACCAGTCGGGTTCCGG		65.79	
*aba*A	F - TCTTCGGTTGATGGATGATTTC	2.02	56.50	Han et al., 2016
	R - CCGTTGGGAGGCTGGGT		61.36	
*brl*A	F – TATCCAGACATTCAAGACGCACAG	1.94	60.92	Chang et al., 2012
	R - GATAATAGAGGGCAAGTTCTCCAAAG		59.52	
wetA	F - CCACAGCAGCCGATCCA	2.05	59.35	Chang et al., 2012
	R - CCCCTTGCAGGATGTCATG		58.20	
β-tubulin	F - TTGAGCCCTACAACGCCACT	2.02	61.76	Wang et al., 2017
	R - TGGTTCAGGTCACCGTAAGAGG		61.67	

The primers efficiencies were calculated from the optimal cDNA dilutions from the given slopes in Bio-Rad CFX Manager software, according to the equation 5.1.

$$(5.1) \qquad E = 10^{\wedge}(-1/slope)$$

The PCR amplification processes were programed as follows: an enzyme activation step at 95ºC for 2 min, a denaturation step at 95ºC for 5 s, an annealing and extension steps at 60ºC for 20 s. Forty amplification cycles of the thermal cycling procedure were run. The specificity of the amplification was confirmed by the melt curve analysis. RT-PCR was performed three times for each sample. Relative expression levels and fold differences were calculated based on the mathematical models from Pfaffl (2001). The fold difference (FD) of a target gene was calculated based on *E* and difference between threshold cycle (Ct) average of the control and the treatment value ΔCt of the treatment sample versus the control sample and expressed in comparison to a reference gene (β-tubulin), according to equation 5.2.

$$FD = \frac{(E_{target})^\wedge(\Delta Ct_{target}\,(control - treatment))}{(E_{ref})^\wedge(\Delta Ct_{ref}\,(control - treatment))} \quad (5.2)$$

The FD of the target gene is expressed in a treatment sample versus a control sample in comparison to a reference gene. E_{target} is the RT-PCR efficiency of the target gene and the E_{ref} is the RT-PCR efficiency of β-tubulin gene. The ΔCt_{target} is the difference between Ct average of the control sample and the treatment sample of the target gene. The ΔCt_{ref} is the difference between Ct average of the control sample and the treatment sample of the β-tubulin gene.

The relative expression was calculated based on the equations 5.3 e 5.4, were the optimal and identical real-time amplification efficiencies of target and reference gene of $E = 2$ were presumed.

$$Relative\ expression = 2^{\Delta\Delta Ct} \quad (5.3)$$

$$\Delta\Delta Ct = \Delta Ct_{ref} - \Delta Ct_{target} \quad (5.4)$$

5.2.4.2. Aflatoxins analysis by high-performance liquid chromatography (HPLC)

Aflatoxins were extracted by adding (1:1, v/v) of the mixture of water:acetonitrile:methanol (3:1:1, v/v/v) into the supernatant recovered at the end of the fermentation. Subsequently, 2 mL of each extraction were filtered through a 0.2 µm syringe filter and analyzed by HPLC in the conditions previously described in Chapter 4.

5.2.5. Transmission electron microscopy (TEM)

Samples were fixed by immersion in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer (pH 7.4) solution for 5 days. After washing and two hours in post-fixating 2% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.4) solution, tissues were washed in buffer, incubated with 1% Uranyl acetate overnight, washed in buffer and dehydrated through graded series of ethanol, and embedded in Epon (EMS). Ultrathin sections were cut at 50 nm and prepared on a RMC Ultramicrotome (PowerTome, USA) using a diamond knife and recovered to 200 mesh Formvar Ni-grids, followed by 2% uranyl acetate and saturated lead citrate solution. Visualization was performed at 80 kV in a (JEOL JEM 1400 microscope (Japan)) and digital images were acquired using a CCD digital camera Orious 1100 W (Tokyo, Japan). The TEM was performed at the HEMS core facility at i3S, University of Porto, Portugal with the assistance of Ana Rita Malheiro e Rui Fernandes.

5.2.6. Statistical analysis

All data were expressed as the means ± standard deviation of four independent replicates. Results were analyzed using the Student´s t-test. All statistical analyses were performed at a significance level of 0.05 using the software IBM SPSS Statistics 23 (IBM Corp., Armonk, USA).

5.3. RESULTS AND DISCUSSION

5.3.1. Conidial production and analysis of the colony morphology by TEM

The amount number of conidia that are produced and released by *Aspergillus* species also contribute to the success of survival of this species. The asexual spores from *Aspergillus* are among the most dominant fungal structure in the air being the most contaminant in agriculture commodities (Teertstra et al., 2017). In the previous chapter, it was found that the rhamnolipids produced by *P. aeruginosa* #112 were able to inhibit at the highest concentration tested, approximately 40% of the *A. flavus* MUM 17.14 growth. During these assays differences in conidia production and hyphae structure in the *A. flavus* MUM 17.14 were observed as the result of the presence of rhamnolipids. At 1.5 g/L of rhamnolipids, the concentration of conidia duplicated as compared to the control (*data not shown*). This appears to be a stress response by *A. flavus* to the presence of rhamnolipids, possibly due to the suppressed or overexpression of the genes involved in conidial development when exposed to rhamnolipids. Han and co-workers (2016) demonstrated that an interference in *nrm*A gene could modify the aflatoxin biosynthesis, conidiation, sclerotia formation, invasive virulence and stress responses. In fact, they demonstrated that the absence of *nrm*A gene in the *A. flavus* strain increases the conidia production comparing to the wild-type. Consequently, Chang and co-workers (2012) demonstrated that a *lae*A deletion (a global regulator) in *A. flavus* strains were responsible for the non-production of sclerotia and for exhibiting developmental abnormalities, like increasing the production of conidiophores, reducing conidial chain elongation, and for a marked reduction in hydrophobicity.

The internal structures of hyphae and conidiophores of *A. flavus* MUM 17.14 were analyzed by TEM. In relation to the spores, TEM images showed that the treated spores (Figure 5.1 C) remained intact and cell components were well arranged similarly to the non-treated ones (Figure 5.1 A). However, it seems that the treated spores exhibit a slight deformation on the spore structure compared to the round shape observed in the non-treated spores. Regarding the hyphae structure, as it can be seen in Figure 5.1 B, the cell-walls of the non-treated *A. flavus* MUM 17.14 fungi are uniform and protected with a uniform shape. It is possible to visualize an intact cell-wall and cell membrane, as well as a good internal organization where it can be seen the organelles including Golgi complex, mitochondria, vacuole with normal appearances. In contrast, in the case of the *A. flavus* MUM 17.14

treated with 1.5 g/L of rhamnolipids, it is possible to observe a detachment from the cell-wall, lysis and disappearance of the plasma membranes, loss of mitochondria, increasing of lipidic content and lack of intracellular content. It has been reported that rhamnolipids interact with the lipid constituents of biological membranes, disturbing their integrity and permeability by inducing the formation of pores and ion channels (Mnif et al., 2015; Sánchez et al., 2007). The pores formation can be seen in Figure 5.1 D, were it is possible to observe the small gaps in the cell-wall of the *A. flavus* MUM 17.14 in the empty hyphae. In fact, Dias and co-workers (2018) showed that amphiphilic chitosan derivates affect the cell-wall integrity by inducing the aggregation of hydrophobic constituents of the conidia from *A. flavus*. Gong and co-workers (2014) demonstrated the antifungal effect of bacillomycin D produced by *Bacillus subtilis* on the mycelium growth, sporulation and spore germination of *A. flavus*. The bacillomycin D was able to injure the cell-wall and cell membrane of the hypha and spore, subsequently cytoplasm and organelles inside the cell were exuded and formed an empty hole. Sangmanee and Hongpattarakere (2014) also observed the same ultrastructure alterations in *A. parasiticus* when treated with components produced by *Lactobacillus plantarum* K35. The rhamnolipids effect on hyphae structure of *A. flavus* MUM 17.14 was stronger than that observed in the conidial structure, this could be due to the thicker cell-walls of the spores.

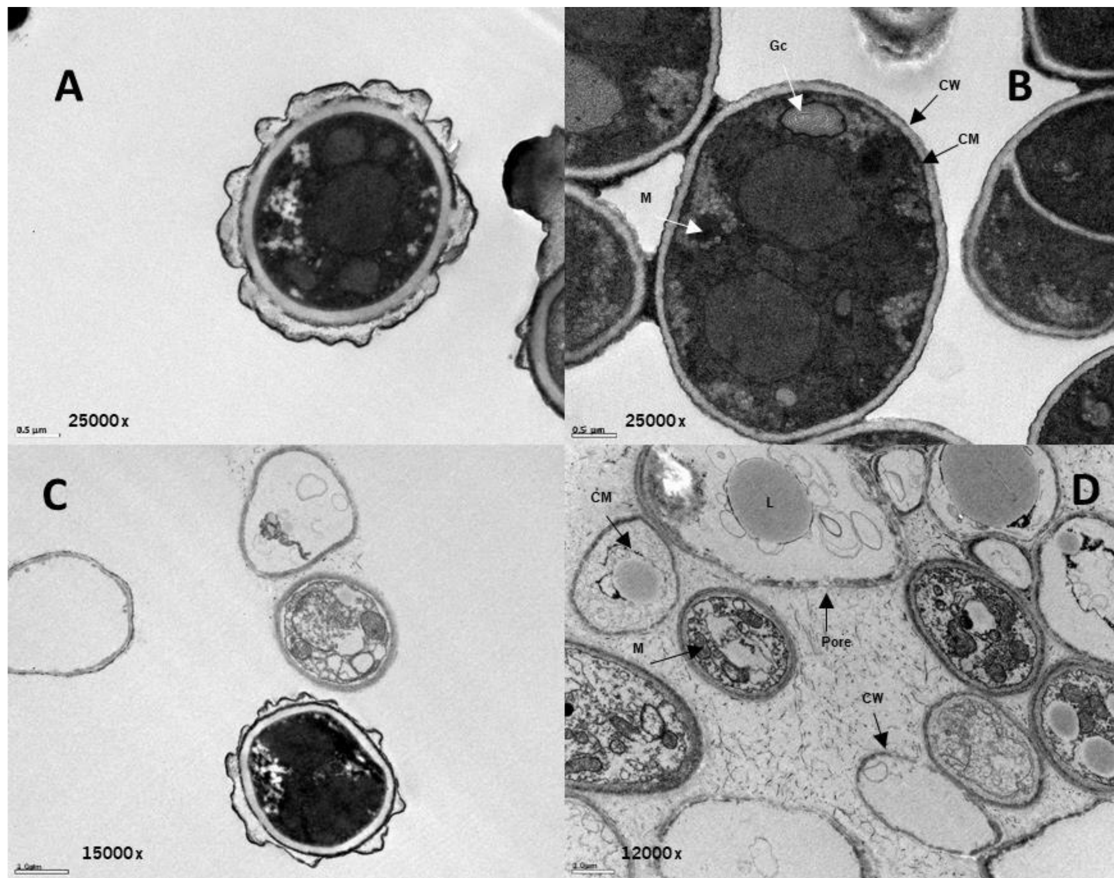

Figure 5.1. Transmission electron microscopy (TEM) sections of *Aspergillus flavus* MUM 17.14 non-treated and treated with 1.5 g/L of rhamnolipids produced by *Pseudomonas aeruginosa* #112, after 5 days at 25ºC: (A, B) non-treated conidiophore and hyphae, (C, D) conidiophore and hyphae treated with 1.5 g/L of rhamnolipids. CW: cell-wall, CM: cell membrane, Gc: Golgi´s complex, M: mitochondria, L: lipid content.

5.3.2. RT-PCR analysis of the expression of genes involved in sporulation

From the TEM images it was possible to observe that rhamnolipids have an effect in the hyphae development, including internal lack or disorganization of the organelles. This could lead to a nutrient deprivation which is the primary signal-inducing sporulation in many microorganisms (Adams and Timberlake, 1990). Genetic studies were performed to evaluate the rhamnolipids effect in *A. flavus* MUM 17.14 germination in order to better understand internal and external changes previously observed. RT-PCR assays were performed to analyze the possible effect of rhamnolipids on central regulatory genes involved in the asexual development. The gene expression of the activator genes of conidiation, namely *brl*A, *aba*A and *wet*A that are involved in the early and terminal stages of conidiophore development, were evaluated. As it can be seen in Figure 5.2, at the

rhamnolipids concentration tested it was possible to observe a 52% reduction of the expression of *brl*A gene. This could lead to the production of elongated stalk and a lack or weak vesicles development or any other subsequent structures (Park and Yu, 2012). The results are in good agreement with the images recorded using TEM (Figure 5.1 D). The exposure to rhamnolipids apparently does not affect the expression of the *aba*A and *wet*A genes. The loss of *aba*A gene function could result in the formation of aberrant conidiophores (Chang et al., 2012). These results are in accordance with the conidial the TEM observations (Figure 5.1 C) in which the conidiophores deformation in samples exposed to rhamnolipids were not observed. The slight irregulated form observed could be due to the delayed expression of *brl*A. The gene *wet*A contributes to the spore integrity and the deletion of *wet*A genes results in the development of conidia with defective cell-walls and no intra-cellular trehalose which leads to the disintegration of spores (Wu et al., 2017). Analyzing the TEM images (Figure 5.1 D), the cell-wall of the spores are well defined which corroborate the RT-PCR results that show no significant differences in the *wet*A expression comparing to the control. The lower expression of *brl*A could be due to the expression of *wet*A since this gene is responsible for the activation and control of the *brl*A gene (Wu et al., 2017).

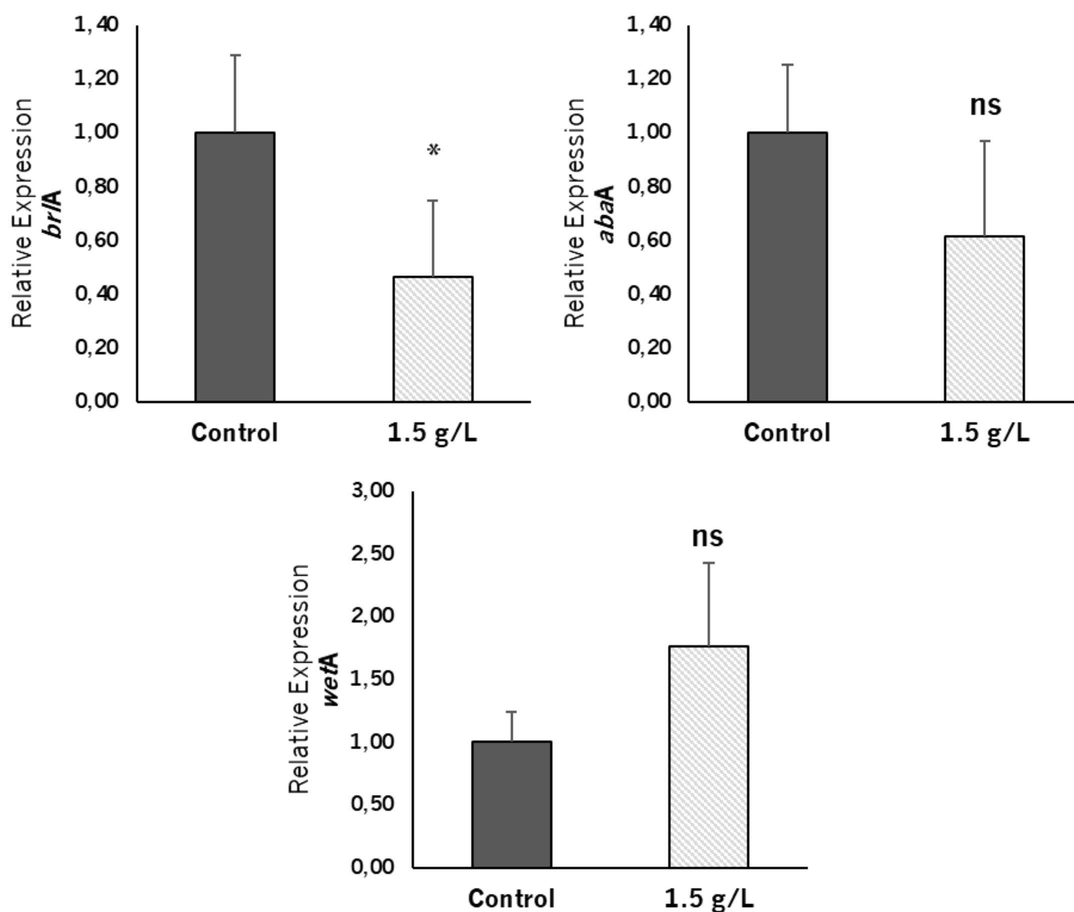

Figure 5.2. Expression of the genes (*brl*A, *aba*A and *wet*A) involved in aflatoxin biosynthesis in *Aspergillus flavus* treated with 1.5 g/L of rhamnolipids produced by *Pseudomonas aeruginosa* #112. The results represent four independent experiments with three replicates each. The *A. flavus* β-tubulin gene was used as an internal control to normalize the expression data. NS denotes no significant differences at $p > 0.05$ and * significant differences at $p < 0.05$ as determined by Student´s t-test.

5.3.3. RT-PCR analysis of the expression of genes involved in aflatoxin biosynthesis

In the previous chapter, it was observed that rhamnolipids, produced by *P. aeruginosa* #112 in solid cultures, inhibit approximately 40% of the *A. flavus* MUM 17.14 growth at the highest concentration tested (1.5 $g_{rhamnolipids}$/L) but completely inhibit the production of aflatoxins. In the liquid cultures, through HPLC analysis, a similar result was observed. The rhamnolipids were able to inhibit approximately 96% of the aflatoxin production.

Based on these results, RT-PCR assays were performed to evaluate the rhamnolipids effect on the gene expression of five genes involved in the aflatoxin production. Among the aflatoxin pathway, the genes *afl*R and *afl*S are the most important ones involved in the pathway regulation. The *afl*R gene is known to be involved in the transcription activation and *afl*S gene is known to be involved in the regulation of aflatoxin biosynthesis (Kong et al., 2010). From the aflatoxins biosynthesis it is known that the *afl*C gene is involved in the first stage of aflatoxin pathway and it is responsible for the synthesis of a polyketide molecule (Yu et al., 2004). Besides, it has been demonstrated that the *afl*Q gene, an oxidoreductase, is necessary for the conversion of O-methylsterigmatocystin (OMST) and dihydro-O-methylsterigmatocystin (DHOMST) into to AFB_1/AFB_2 and AFG_1/AFG_2, respectively (Yu, 2012). As it can be seen in Figure 5.3, the treatment with rhamnolipids suppressed the *alf*C and *afl*Q expression by 85% and 50%, respectively. However, the rhamnolipids did not affect the *afl*R and *afl*S expression.

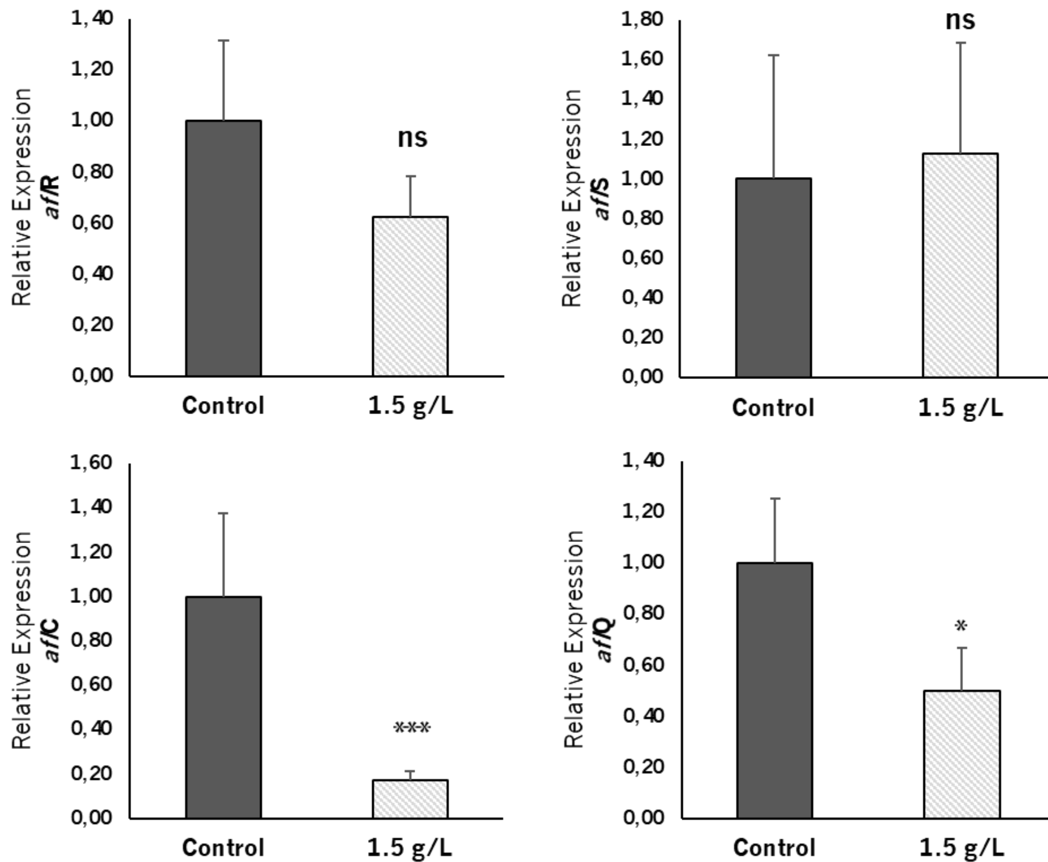

Figure 5.3. Expression of the genes (*afl*R, *afl*S, *afl*C and *afl*Q) involved in aflatoxin biosynthesis in *Aspergillus flavus* treated with 1.5 g/L of rhamnolipids produced by *Pseudomonas aeruginosa* #112. The results represent four independent experiments with three replicates each. The *A. flavus* β-tubulin gene was used as an internal control to normalize the expression data. NS denotes no significant differences at $p > 0.05$, * significant differences at $p < 0.05$ and *** significant differences at $p < 0.001$ as determined by Student´s t-test.

These results are in accordance with previous works. Wang and co-workers (2017) tested different nitrogen sources to evaluate their effect on the aflatoxin biosynthesis. The different nitrogen sources promoted the aflatoxin production. The *afl*R expression did not change under the different nitrogen sources tested, however the *afl*S gene was up-regulated in the glutamine source. Kong and co-workers (2010) demonstrated that the marine bacteria *Bacillus megaterium* presents antifungal activity against *A. flavus* and was able to inhibit the biosynthesis of aflatoxin. The *B. megaterium* suppressed the expression of the *afl*R and

*afl*S gene, which could be related with a decrease of the aflatoxin production. Moon and co-workers (2018) tested five different organic acids used as food additives to evaluate their effect in the aflatoxin biosynthesis. The authors demonstrated that some of the organic acids, such as benzoic acid, butyric acid and acetic acid were able to suppress the *afl*C, *afl*Q, *afl*R and *afl*S expression. Regardless of the suppressed genes, not all these organic acids were able to inhibit the aflatoxin production from *A. flavus*. This was the first time that rhamnolipids effect on aflatoxin biosynthesis in *A. flavus* was evaluated. Despite the fact that the aflatoxin biosynthesis process and a clustered pathway of genes associated have been known for more than a decade, more studies ought to be performed to fully understand the mechanisms underlying the inhibition of aflatoxin production.

5.4. CONCLUSIONS

Rhamnolipids produced by *P. aeruginosa* #112 in CSLM medium completely inhibit the aflatoxin production by *A. flavus*, comprising a promising “green” alternative to control the *A. flavus* growth and the concentration of aflatoxin present in different agriculture commodities. Exposure to rhamnolipids highlighted external and internal morphologic alterations in *A. flavus* hyphae with irreversible damage making unfeasible the fungus growth. In addition, the presence of rhamnolipids was found to suppress the expression of the genes *afl*C and *afl*Q from the aflatoxin pathway, which could explain the aflatoxin inhibition among the several damages observed in the internal content of the *A. flavus* hyphae.

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CHAPTER 6

ENGINEERING *PSEUDOMONAS AERUGINOSA* STRAINS TOWARDS THE PRODUCTION OF DI-RHAMNOLIPIDS

ABSTRACT

Rhamnolipids are a good alternative to replace the chemical surfactants for industrial and environmental applications due to their attractive properties. One of the strategies to increase the rhamnolipids competitiveness comprises the engineering of a "novel" strain able to produce high yields of rhamnolipids or the most efficient rhamnolipid congener for a given purpose. *Pseudomonas aeruginosa* is known to be the best rhamnolipids producer and the study of the complex regulation network key genes involved in their biosynthesis remains a challenge. In this study, the *rhl*C gene, cloned from *P. aeruginosa* #112, was used to construct the recombinant plasmid pVLT31_PsRhlC (with a strong promotor induced by ITPG) in order to increase the di-rhamnolipids production (previously found to exhibit the best anti-fungal activity). In addition, the *rhl*C gene with its native promotor (PA1131) was amplified from *P. aeruginosa* #112 and cloned into the plasmid pBBR1MCS-5 to construct the recombinant plasmid pBBR1MCS-5_PRhlC. The strain *P. aeruginosa* #2 was transformed with both recombinant plasmids. Both transformants with the recombinant plasmids, at the optimal conditions, exhibited an increase of the di-rhamnolipids production when compared with the wild type, being the transformant with the recombinant plasmid pVLT31_PsRhlC the most efficient. These results demonstrated that the engineered strain enhanced the production of the di-rhamnolipids in *P. aeruginosa*, suggesting its potential to be used in industrial sustainable bioprocesses.

Keywords: Di-rhamnolipids, *rhl*C, pVLT31, pBBR1MCS-5, *P. aeruginosa*

CONTENTS

6.1. INTRODUCTION 147

6.2. MATERIAL AND METHODS 148

6.2.1. BACTERIAL STRAINS, PLASMIDS AND CULTURE CONDITIONS 148

6.2.2. GENOMIC DNA EXTRACTION AND AMPLIFICATION OF GENE *RHL*C 149

6.2.3. CONSTRUCTION OF RECOMBINANT PLASMIDS 150

6.2.4. *PSEUDOMONAS AERUGINOSA* TRANSFORMATION (ELECTROPORATION)…… 152

6.2.5. RHAMNOLIPIDS PRODUCTION AND RECOVERY 152

6.2.6. SURFACE TENSION AND CRITICAL MICELLAR CONCENTRATION (*CMC*)…… 152

6.2.7. RHAMNOLIPIDS CHARACTERIZATION 153

6.3. RESULTS AND DISCUSSION 153

6.3.1. EFFECT OF THE EXPRESSION OF *RHL*C IN THE RHAMNOLIPIDS PRODUCTION IN *P. AERUGINOSA* TRANSFORMANTS 153

6.3.2. CHARACTERIZATION OF THE RHAMNOLIPIDS MIXTURES PRODUCED BY *P. AERUGINOSA* TRANSFORMANTS 157

6.4. CONCLUSIONS 160

6.5. REFERENCES 160

6.6. SUPPLEMENTARY MATERIAL 166

6.1. INTRODUCTION

Surfactants are amphiphilic molecules, mainly petroleum derived, which accumulate between the interface of polar and non-polar phases. Due to this property, they are the most adaptable compounds used in the chemical industry, being used in every industrial area from household detergents and food items to pesticides and pharmaceuticals (Vaz et al., 2012). Due to the advances in biotechnology and the increasing environmental concerns, the "green surfactants", namely biosurfactants, gained an increased attention in the past years (Bahia et al., 2018). Despite of their advantageous properties such as high biodegradability, low toxicity and high performance at extreme conditions, the main disadvantage that make them less attractive and economically viable as compared to their chemical analogues is the high cost associated to their production (Bharali et al., 2013; Gudiña et al., 2015; Nalini and Parthasarathi, 2014). The low yields, the cost of culture medium and the downstream processing make them expensive (Banat et al., 2014). One of the strategies to improve the biosurfactants production could include the design and construction of 'new strains' with higher yields. For that purpose, the study of the genetic regulation and biosynthesis of biosurfactants is crucial (Lovaglio et al., 2015).

From the vast diversity of biosurfactants, rhamnolipids are the most studied. Indeed, up to the current year the highest number of patents on biosurfactants registered are related with the glycolipids class (EPO, 2019). Rhamnolipids are mainly produced by *Pseudomonas* and *Burkholderia* species being *Pseudomonas aeruginosa* the best producer (Rodrigues et al. , 2017). They are usually produced as a complex mixture of congeners compounds of one or two molecules of dTDP-L-rhamnose linked to a 3- hydroxyalkanoic acid dimer (Dubeau et al., 2009). The most abundant congeners produced by *P. aeruginosa* are the mono-rhamnolipid (Rha-C_{10}-C_{10}) and the di-rhamnolipid (Rha-Rha-C_{10}-C_{10}) (Sha et al., 2012). The studies of the rhamnolipids biosynthetic pathway have demonstrated the implication of three crucially important genes, namely *rhl*A, *rhl*B and *rhl*C (Dubeau et al., 2009). These genes are transcribed in the same direction, with *rhl*AB being responsible for the mono-rhamnolipids synthesis and *rhl*C responsible for di-rhamnolipids synthesis (Dubeau et al., 2009; Rahim et al., 2000; Rahim et al., 2001). The *rhl*A gene is responsible for the formation of the hydrophobic portion using the ACP-β-hydroxyacids as substrate (Chong and Li, 2017; Dubeau et al., 2009). The hydrophobic portion is used as substrate, as well as the dTDP-L-rhamnose for the enzyme rhamnosyltransferase *rhl*B catalyzing the

formation of mono-rhamnolipids (Dubeau et al., 2009; Soberón-Chávez et al., 2005). The rhamnosyltransferase II *rhl*C, located in another region of the chromosome, is responsible for the incorporation of the second dTDP-L-rhamnose to a mono-rhamnolipid forming the di-rhamnolipid (Wittgens et al., 2011). For many applications it would be desirable to have only one congener instead of mixture (Müller et al., 2012). Several studies attributed the antifungal activity to the congener di-rhamnolipid that could be a good "green" alternative to replace the chemical pesticides. In fact, Rodrigues and co-workers (2017) demonstrated that di-rhamnolipids at lower concentrations together with an optimal NaCl concentration, present a higher antifungal activity against *Aspergillus niger* and *Aspergillus carbonarius* when compared to the congener mono-rhamnolipids. Also, in Chapter 4, it was reported that the di-rhamnolipids produced by *P. aeruginosa*, at low concentrations, present more antifungal activity than the mono-rhamnolipids and completely inhibit the aflatoxins production by *Aspergillus flavus*. In addition, Sha and co-workers (2012) also reported that di-rhamnolipids from *P. aeruginosa* exhibit higher antifungal activity against different plant pathogens when compared with mono-rhamnolipids.

The *Burkholderia* species, such as *Burkholderia pseudomallei* and *Burkholderia thailandensis* have been described as mainly producers of long-chain di-rhamnolipids predominately composed of a C_{14}-C_{14} chain length fatty acid moiety (70 to 77% of total rhamnolipids), following by others comprising C_{10}-C_{12} and C_{16}-C_{16} (Dubeau et al., 2009; Elshikh et al., 2017).

The aim of this chapter was to construct *P. aeruginosa* #2 derivatives with the purpose of increasing the di-rhamnolipids production. This strain produced high levels of a mixture of mono- and di-rhamnolipids. Three different plasmids constructions were made using the *rhl*C gene amplified from *P. aeruginosa* #112 and *B. thailandensis* E264. Furthermore, the rhamnolipids produced by *P. aeruginosa* derivatives were chemically characterized.

6.2. MATERIAL AND METHODS

6.2.1. Bacterial strains, plasmids and culture conditions

Luria Bertani (LB; NaCl 10 g/L; tryptone 10 g/L; yeast extract 5 g/L; pH 7) medium was used for the strains *P. aeruginosa* #112 and *P. aeruginosa* #2 and Nutrient Broth (NB; peptone 5 g/L; meat extract 3 g/L; pH 7) medium was used for the strain *B. thailandensis*

E264 (DSM 13276). The following plasmids were used in this study: pVLT31 (de Lorenzo et al., 1993) and pBBR1MCS-5 (obtained from Westerdijk Fungal Biodiversity Institute, NCCB collection ((NCCB 3437) (Kovach et al., 1995)). The plasmids were propagated in, and isolated from *Escherichia coli* NZY5α (Nzytech genes & enzymes). For selection of *E. coli* and *P. aeruginosa* transformants, antibiotics were added to LB agar plates at final concentrations, respectively: tetracycline (Tc) 10 and 50 µg/mL (NZYTech) and gentamycin (Gm) 10 µg/mL (NZYTech). When necessary, isopropyl-β-D-thiogalactopyranoside ((IPTG), 0.4 mM) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 20 µg/mL) were added to the medium. The *E. coli, P. aeruginosa* #112 and *P. aeruginosa* #2 strains were grown at 150 rpm at 37ºC. The *B. thailandensis* E264 strain was grown at 180 rpm and 30ºC. The bacterial strains were stored at -80ºC in the respective medium until further use.

6.2.2. Genomic DNA extraction and amplification of gene *rhl*C

The gene responsible for the biosynthesis of di-rhamnolipids (*rhl*C) was amplified from the genomic DNA of *P. aeruginosa* #112 and *B. thailandensis* E264 isolated with Wizard® Genomic DNA Purification Kit (Promega Corporation). The genomic DNA was quantified by determining the absorbance at 260 and 280 nm using a Nanodrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fischer Scientific Inc). The genomic DNA quality was evaluated through agarose gel (0.8%, w/v) electrophoresis. Three separate PCR amplifications of this *rhl*C region were performed. The reverse primer used in each reaction was tagged with *Hin*dIII restriction sequence. In each case, the forward primer was tagged with a restriction enzyme recognition site and the beginning of the *rhl*C sequence. In the case of the plasmid pBBR1MCS-5 it was necessary to include the gene PA1131 that is located upstream of the *rhl*C gene sequence in the same operon, since this plasmid does not contain a promotor region. The sequences of the primers (synthesized by Metabion International and Thermo Fisher Scientific Inc) and restriction sites used for cloning are listed in Table 6.1. The PCR cycle conditions were the following: initial denaturation at 98ºC for 1 min, followed by 30 cycles of a denaturation step at 98ºC for 10 s, annealing and extension steps with different temperatures and times according to the primers used and when necessary dimethyl sulfoxide (DMSO) was add (also listed in Table 6.1); and a final extension at 72ºC for 10 min. The first primers were used to amplify the *rhl*C gene from the genomic DNA obtained from the different strains and the second primers were

constructed to amplify directly from the *rhl*C gene recovered from the genomic DNA. To amplify the *rhl*C gene, the Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientifc Inc) was used according to the kit specifications. All *rhl*C PCR products were recovered with NucleoSpin® Gel and PCR clean-up Kit (MACHEREY-NAGEL GmbH & Co.) and quantified through measures of the absorbance at 260 and 280 nm using a Nanodrop ™ One Microvolume UV-Vis Spectrophotometer.

6.2.3. Construction of recombinant plasmids

After sequence confirmation (Supplementary material), each amplified product was digested with the respective restriction enzymes as described in Table 6.1. The plasmid pVLT31 was digested with *Xab*I and *Hin*dIII and the plasmid pBBR1MCS-5 was digested with *Hin*dIII, following the manufacturer's instructions (ThermoFisher Scientific, Inc). The three *rhl*C PCR products digested were ligated in the respective sites of the plasmids by using T4 DNase ligase according to the protocol provided by the manufacturer (ThermFisher Scientific, Inc). The plasmid pVLT31 was ligated with *rhl*C PCR product digested with the two restriction enzymes, amplified from *P. aeruginosa* #112 and *B. thailandensis* E264; the different plasmids were designated pVLT31_PsRhlC and pVLT31_BRhlC, respectively. The PA1131-*rhl*C PCR product digested only with *Hin*dIII was ligated to the plasmid pBBR1MCS-5, namely pBBR1MCS-5_PRhlC. The different recombinant plasmids were propagated in, and isolated from *E. coli* NZY5α. *E. coli* NZY5α cells containing the plasmids pVLT31_PsRhlC and pVLT31_BRhlC were selected by adding 10 µg/mL of Tc into the LB agar medium after incubation at 37ºC. The different clones containing the respective recombinant plasmids were selected using agarose gel (0.8%, w/v) electrophoresis according to the different DNA length of the different recombinant plasmids, the DNA length for the plasmid pVLT31_PsRhlC is 10998 bp and the DNA length for the plasmid pVLT31_BRhlC is 10985 bp. *E. coli* NZY5α cells containing the recombinant plasmid pBBR1MCS-5_PRhlC were selected by adding 10 µg/mL of Gm and 20 µg/mL of X-Gal into the LB agar plates after incubation at 37ºC. For confirmation purposes, the different recombinant plasmids from the different selected clones, were recovered and digested with the respective restriction enzymes and were further verified through agarose gel (0.8%, w/v) electrophoresis and sequencing (GATC Biotech, Konstanz, Germany). All the plasmids from the different colonies were recovered by using MiniPrep Kit or MaxiPrep Kit (ThermFisher Scientific, Inc) whenever necessary.

Table 6.1. PCR primers, primer melting temperature (Tm), restriction enzymes and PCR conditions used to amplify the *rhl*C gene, and length of products obtained for the strains *Pseudomonas aeruginosa* #112 and *Burkholderia thailandensis* E264

Strain	Primer	Tm (^{O}C)	Sequence (5'→ 3')	Restriction enzymes	PCR conditions	Length
P. aeruginosa #112	RhlC_F	71.5	TTTTTCTAGACCTACGGGAGAAGAACGATCATGGACCG	*Xba*I	Annealing 72ºC for 15s Extension 72ºC for 30s	998 bp
	RhlC_R	71.7	TTTAAGCTTCTAGGCCTTGGCCTTGCCGG	*Hin*dIII		
	RhlC2_F	52.1	TTTTTCTAGACCTACGGG	*Xba*I	Annealing 54ºC for 15s Extension 72ºC for 30s	998 bp
	RhlC2_R	52.1	TTTAAGCTTCTAGGCCTT	*Hin*dIII		
	PRhlC_F	67.2	TTTAAGCTTTCGCATCATTATGACATCACGCC	*Hin*dIII	Annealing 72ºC for 15s Extension 72ºC for 1min + 3% of DMSO	2370 bp
	RhlC_R	71.7	TTTAAGCTTCTAGGCCTTGGCCTTGCCGG	*Hin*dIII		
	PRhlC2_F	51.4	TTTAAGCTTTCGCATCAT	*Hin*dIII	Annealing 51.3 ºC for 15s Extension 72ºC for 45s + 3% of DMSO	2370 bp
	RhlC2_R	52.1	TTTAAGCTTCTAGGCCTT	*Hin*dIII		
B. thailandensis E264	BRhlC_F	76.5	TTTTTCTAGAAACCACCAAAGGGAAGAGCGATGAC	*Xba*I	Annealing 72ºC for 30s Extension 72ºC for 30s	985 bp
	BRhlC_R	77.6	TTTAAGCTTTCATTCCTGCCGCACCGG	*Hin*dIII		
	BRhlC2_F	50.1	TTTTTCTAGAACCACCAA	*Xba*I	Annealing 62ºC for 30s Extension 72ºC for 30s + 10% of DMSO	985 bp
	BRhlC2_R	53.2	TTTAAGCTTTCATTCCTG	*Hin*dIII		

6.2.4. *Pseudomonas aeruginosa* transformation (Electroporation)

P. aeruginosa #2 was transformed through electroporation with the three different plasmids constructed (and with the respective empty plasmids for control purposes) according to Choi et al. (2006). The different *P. aeruginosa* transformants containing the plasmids pVLT31_PsRhlC and pVLT31_BRhlC were selected by adding 50 µg/mL of Tc into the LB agar medium after incubation at 37ºC. The recombinant *P. aeruginosa* containing the plasmid pBBR1MCS-5_PRhlC was selected by adding 10 µg/mL of Gm into the LB agar plates after incubation at 37ºC. Different clones were selected to further evaluate the production of di-rhamnolipids.

6.2.5. Rhamnolipids production and recovery

The different clones selected were grown in shake flasks containing 50 mL of LB medium and 50 µg/mL of Tc, for pVLT31 transformants, and 10 µg/mL of Gm, for the strain pBBR1MCS-5 in order to evaluate the rhamnolipids production profile. For the plasmids pVLT31, in order to induce the expression of the *rhl*C gene it is necessary to add IPTG to the medium. Therefore, different IPTG concentrations (0.0, 0.2, 0.4, 0.8 and 1.0 mM) were evaluated to find the concentration leading to the higher expression. Each flask was inoculated with 500 µL of pre-culture that was prepared at the same culture medium conditions and incubated overnight. The flasks were then incubated at 37ºC at 180 rpm for 3 days. At the end of the fermentation, cells were harvested by centrifugation (10000 x g for 20 min). In order to reduce the number of clones and identify the best di-rhamnolipids producers, a pre-selection was performed through thin layer chromatography (TLC) as described in Chapter 3. The rhamnolipid mixtures produced by the 'best' clones were recovered by adsorption chromatography using the polystyrene resin Amberlite XAD-2 (Sigma-Aldrich Co., USA) as previously described in Chapter 2. The rhamnolipids production was determined as dry-weight of the freeze-dried purified products.

6.2.6. Surface tension and critical micellar concentration (*cmc*)

The surface tension was measured by the Ring method described in Chapter 2 using a KRÜSS K20 Tensiometer (KRÜSS GmbH, Germany) equipped with a 1.9 cm De Noüy platinum ring at room temperature (25ºC). All measurements were performed in triplicate. The *cmc* were calculated as described in Chapter 2, using the freeze-dried rhamnolipids

produced by the different *P. aeruginosa* recombinants, dissolved in demineralized water at different concentrations.

6.2.7. Rhamnolipids characterization

In order to characterize the rhamnolipids produced by the different recombinant *P. aeruginosa* strains, several samples were analyzed by electrospray ionization (ESI) mass spectrometry (MS) as described previously in Chapter 2.

6.3. RESULTS AND DISCUSSION

6.3.1. Effect of the expression of *rhl*C in the rhamnolipids production in *P. aeruginosa* transformants

The rhamnolipids biosynthesis in *P. aeruginosa* occurs in three consecutive enzymatic reactions, where the genes *rhl*A plus *rhl*B and *rhl*C are responsible for the mono- and di-rhamnolipids biosynthesis, respectively (Wittgens et al., 2016). The *B. thailandensis* E264 is another microorganism reported as a rhamnolipid producer, for which the genes *rhl*A, *rhl*B and *rhl*C are organized in a single gene cluster, whereas in *P. aeruginosa* the gene *rhl*C is separated from the *rhl*A and *rhl*B genes (Irorere et al., 2017). In the previous chapters, it was demonstrated that the di-rhamnolipid congeners produced by *P. aeruginosa* exhibit, at lower concentrations, a higher antifungal activity and anti-aflatoxin production compared to the mono-rhamnolipids. In order to increase the di-rhamnolipids production by *P. aeruginosa* #2, three different plasmids carrying the *rhl*C gene (from *P. aeruginosa* #112 and *B. thailandensis* E264) that is responsible for adding the second molecule of rhamnose to the mono-rhamnolipids, thus forming the di-rhamnolipids molecules, were tested. The plasmids pVLT31 and pBBR1MCS-5, used for the different transformations, are broad vectors that can be used in several hosts, and have been tested and found to be stable in *P. aeruginosa* (de Lorenzo et al., 1993; King et al., 2008; Kovach et al., 1995; Wilderman et al., 2002; Zhao et al., 2015). *B. thailandensis* E264 produces predominantly di-rhamnolipids with C_{14}-C_{14} fatty acid chains (Dubeau et al., 2009; Funston et al., 2016). One of the strategies tested to increase the production of di-rhamnolipids by *P. aeruginosa* #2 was the expression of the *rhl*C gene amplified from *B. thailandensis* E264. The *P. aeruginosa* transformants with the recombinant plasmid pVLT31_BRhlC exhibited high surface tension values and very low yields (*data not shown*) when compared with the wild

type *P. aeruginosa* #2. Besides, the TLC analysis showed that the rhamnolipids production profile was very similar to the wild type *P. aeruginosa* #2 (Supplementary material, Figure 6.S1). It was not possible to observe an increase of di-rhamnolipids production using these transformants and, consequently, other strategies using the recombinant plasmids pVLT31_PsRhlC and pBBR1MCS-5_PRhlC (where the *rhl*C gene was amplified from *P. aeruginosa* #112) were implemented and were found to be more promising.

In the first approach, the rhamnolipids production profile from the *P. aeruginosa* holding the plasmid pVLT31_PsRhlC was evaluated. The optimal concentration of IPTG to induce the expression of *rhl*C was studied. Different concentrations of IPTG (0.0, 0.2, 0.4, 0.8 and 1.0 mM) were tested and at the end of each fermentation the profile of the rhamnolipids produced by the different transformants pVLT31_PsRhlC was analyzed through TLC (Figure 6.1) and surface tension measurements.

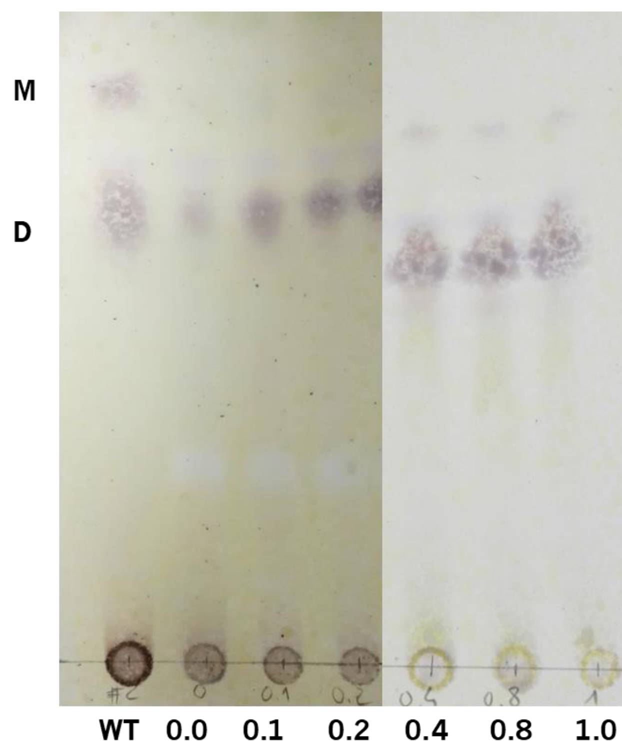

Figure 6.1. TLC of the mono- (**M**) and di-rhamnolipids (**D**) profile at the end of the fermentation at 37ºC at 180 rpm by *P. aeruginosa* #2 (**WT**) and the transformants of *P. aeruginosa* with pVLT31_PsRhlC with different concentrations of IPTG (0.0, 0.1, 0.2, 0.4, 0.8 and 1.0 mM of IPTG).

These results confirmed the need to add ITPG to the culture medium to induce and increase the di-rhamnolipids production by pVLT31_PsRhlC transformants. The fermentations run without IPTG (control) and with the lowest concentrations studied (0.1 and 0.2 mM) showed similar di-rhamnolipids production as compared to the wild type *P. aeruginosa* #2. On the other hand, in the case of the fermentations conducted with the higher IPTG concentrations (0.4, 0.8 and 1.0 mM), the transformants showed higher di-rhamnolipids production comparing to the wild type. An increase of the di-rhamnolipids stain and a decrease of the mono-rhamnolipids stain was observed in the TLC plate comparing to the wild type. Consequently, and bearing in mind the goal of decreasing the production costs, an optimal ITPG concentration of 0.4 mM was chosen. These results are in good agreement with other reports. Wittgens and co-workers (2016) used 0.4 mM of ITPG in the culture medium to induce the expression of the different genes from *Pseudomonas putida* KT2440 transformants holding pVLT33_*rhl*ABC and pVLT33_*rhl*AB plasmids. Aguirre-Ramírez and co-workers (2012) used a higher concentration of IPTG (1 mM) on culture medium to increase the rhamnolipids production from the different *P. aeruginosa* PAO1 transformants.

Furthermore, in the current work the rhamnolipid production profile from the *P. aeruginosa* #2 transformants holding the pBBR1MCS-5_PRhlC plasmid, where the promotor used to induce the expression of the *rhl*C gene was the native promotor PA1131 amplified together with the *rhl*C gene (both genes at the same operon) from the genomic DNA of *P. aeruginosa* #112, was evaluated (Rahim et al., 2001). After finding the best transformant for each recombinant plasmid, the rhamnolipid production was evaluated and compared with the wild type and with the transformants with the empty plasmids (i.e. without the *rhl*C) at the same conditions (Table 6.2).

Table 6.2. Surface Tension values (ST, mN/m), Surface Tension diluted 10 times with demineralized water (ST^{-1}, mN/m), rhamnolipid production ([RL], g/L) and critical micelle concentrations (*cmc*, mg/L) obtained with *Pseudomonas aeruginosa* #2 and the different transformants grown in LB medium at the optimal fermentation time. Results represent the average of three independent experiments ± standard deviation.

Pseudomonas aeruginosa	**Time (h)**	**ST (mN/m)**	**ST^{-1} (mN/m)**	**[RL] g/L**	***cmc* (mg/L)**
P. aeruginosa #2 (WT)	96	32.9 ± 0.1	46.3 ± 0.5	1.203 ± 0.244	30.3
pVLT31	96	30.8 ± 0.0	49.1 ± 0.9	1.051 ± 0.199	-
pVLT31 + 0.4 mM IPTG	96	30.0 ± 0.1	48.8 ± 1.0	1.017 ± 0.231	-
pVLT31_PsRhlC	96	30.8 ± 0.2	47.1 ± 0.3	0.918 ± 0.123	-
pVLT31_PsRhlC + 0.4 mM IPTG	96	32.6 ± 0.2	48.0 ± 1.0	1.217 ± 0.221	500
pBBR1MCS-5	72	34.7 ± 0.1	52.7± 1.0	0.668 ± 0.130	-
pBBR1MCS-5_PRhlC	72	32.1 ± 0.1	46.0 ± 0.6	0.847 ± 0.123	375

As shown in Table 6.2, the yield of rhamnolipids reached the maximum at 96 h for the wild type strain and the engineered strains with the different recombinant pVLT31 plasmids, and at 72 h for the engineered strains with the recombinant pBBR1MCS-5 plasmids. Despite the fact that the engineered strains with recombinant pBBR1MCS-5 plasmids required less hours to achieve the maximum rhamnolipid production, the yields were found to be lower when compared with the wild type strain and the engineered strains with the recombinant pVLT31 plasmids that exhibited yields similar to the wild type strain. The *cmc* value of the engineered strains pBBR1MCS-5_PRhlC was lower than the *cmc* found for the engineered strain pVLT31_PsRhlC (with 0.4 mN of IPTG), but both values are higher than the ones obtained for the wild type. These results could suggest a higher di-rhamnolipids production by the engineered strains. As reported in the previous chapters, the presence of more hydrophilic congeners such as di-rhamnolipids and mono-rhamnolipids with only one fatty acid exhibit higher *cmc* (up to 200 mg/L) and more hydrophobic ones like mono-rhamnolipids with two fatty acids show lower *cmc* values (5 to 40 mg/L) (Abdel-Mawgoud et al., 2009; Bharal and Konwar, 2011; Haba et al., 2003; Sarachat et al., 2010; Zhang et al., 2014; Zhang et al., 2008). The surface tension values from the engineered strains with the recombinant plasmids pVLT31_PsRhlC (with 0.4 mM of ITPG) and pBBR1MCS-5_PRhlC were very similar comparing to the wild type strain. On the other hand, from the engineered strains with the plasmids, pVLT31, pVLT31 with 0.4 mM of ITPG and pVLT31_PsRhlC without IPTG exhibit lower surface tension values without dilution, which could indicate a higher production of mono-rhamnolipids. At the end of each fermentation, the *P. aeruginosa* colonies were inoculated on fresh LB plates containing 50 µg/ml of Tc for the plasmid pVLT31 and 10 µg/mL of Gm for the plasmid pBBR1MCS-5 in order to confirm the stability of the recombinant plasmids.

6.3.2. Characterization of the rhamnolipids mixtures produced by *P. aeruginosa* transformants

The rhamnolipid mixtures produced by the wild type and engineered strains with the different recombinant plasmids were characterized by mass spectrometry by direct injection in an Orbitrap ESI-MS spectrometer operating in the negative mode. The measured mass of the $[M-H]^-$ pseudomolecular ions was matched with exact mass of the molecular structures expected for mono- and di-rhamnolipids, based on similar studies

previously reported (Gudiña et al., 2015). According to Table 6.3, in all cases three different rhamnolipids congeners were identified.

The wild type strain *P. aeruginosa* #2 produced mostly the mono-rhamnolipid Rha-C_{10}-C_{10} (58.4%), followed by the di-rhamnolipid Rha-Rha-C_{10}-C_{10} (31.0%) and the di-rhamnolipid Rha-Rha-C_{10}-C_{12} (10.6%). The transformants with the recombinant plasmid pVLT31_PsRhlC without addition of ITPG to the culture medium exhibit similar rhamnolipid production profiles to the wild type strain, being the most abundant congener the mono-rhamnolipid Rha-C_{10}-C_{10} (58.2%). The transformants with the recombinant plasmid pVLT31_PsRhlC using 0.4 mM of IPTG were found to produce more di-rhamnolipids (61.9%), being the most abundant congener the di-rhamnolipid Rha-Rha-C_{10}-C_{10} (50.5%) followed by the mono-rhamnolipid Rha-C_{10}-C_{10} (38.1%) and the di-rhamnolipid Rha-Rha-C_{10}-C_{12} (11.3%). These results corroborate the need of using ITPG to induce the expression of the *rhl*C gene to increase the production of di-rhamnolipids.

Table 6.3. Relative abundance (%) of rhamnolipids produced by *Pseudomonas aeruginosa* #2 (WT) and the different transformants, obtained at the end of the fermentation performed in flasks, identified as $[M-H]^-$ ions by mass spectrometry.

		Pseudomonas aeruginosa						
Rhamnolipid Congener	**$[M-H]^-$**	**#2 (WT)**	**pVLT31**	**pVLT31+ 0.4 mM IPTG**	**pVLT31_PsRhlC**	**pVLT31_PsRhlC +0.4 mM IPTG**	**pBBR1MCS-5**	**pBBR1MCS-5_PRhlC**
Rha-C_{10}-C_{10}	503.32234	58.4 %	88.8 %	93.7 %	58.2 %	38.1 %	54.7 %	38.9 %
Rha-C_{10}-C_{12}	531.35332	-	-	-	-	-	11.8 %	10.3 %
Rha-Rha-C_{10}-C_{10}	649.38051	31.0 %	7.4 %	4.5 %	31.7 %	50.5 %	33.5 %	50.8 %
Rha-Rha-C_{10}-C_{12}	677.41140	10.6 %	3.8 %	1.8 %	10.1 %	11.3 %	-	-
Ratio mono-/di-rhamnolipids	-	**1.40**	**7.92**	**14.87**	**1.39**	**0.62**	**1.98**	**0.97**

However, an interesting result was the one obtained for the transformants with the recombinant plasmids pVLT31 and pVLT31 using 0.4 mM of IPTG, where the most abundant congener was the mono-rhamnolipid Rha-C_{10}-C_{10}, 88.8% and 93.7%, respectively. The high concentration of mono-rhamnolipids could justify the lower surface tension values observed for these transformants (Table 6.2). Comparing the rhamnolipids production profile from the transformant pVLT31 with the transformant with pVLT31_PsRhlC, both using 0.4 mM of IPTG, a decrease of 59.8% of total mono-rhamnolipids produced was observed. In the case of the recombinant plasmid pBBR1MCS-5, the transformants produced two mono-rhamnolipid congeners, being the most abundant the Rha-C_{10}-C_{10} (54.7%) and the Rha-C_{10}-C_{12} (11.8%) and one type of di-rhamnolipid (33.5%). The transformants with the recombinant plasmid pBBR1MCS-5_PRhlC showed a similar total mono- and di-rhamnolipids production, 49.2% and 50.8%, respectively. According to these results, the transformants with the recombinant plasmid pVLT31_PsRhlC induced with 0.4 mM of ITPG was found to be the most promising engineered *P. aeruginosa* strain regarding the production of di-rhamnolipids.

6.4. CONCLUSIONS

In order to improve the di-rhamnolipid production by *P. aeruginosa* #2, the gene *rhl*C was cloned in two different plasmids, pVLT31 and pBBR1MCS-5. The recombinant plasmid pVLT31_PsRhlC with the optimum IPTG concentration (0.4 mM) was found to be the best vector to engineer *P. aeruginosa* #2 towards a higher production of di-rhamnolipid congeners. The promotor induced by IPTG showed to be stronger than the native promotor PA1131 from *P. aeruginosa*. Overall, the results gathered in this work clearly highlight that additional studies are required to further improve the engineered "novel" *P. aeruginosa* towards an optimized production of di-rhamnolipids that could be scalable to industry.

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6.6. SUPPLEMENTARY MATERIAL

Sequencing results

1. Complete sequence of rhamnosyltransferase II (*rhl*C) from *Pseudomonas aeruginosa* #112, product length of 998 bp.

CTAGGCCTTGGCCTTGCCGGAAGCTACGGACGCCACGGCCGGGCCGGCGAGG
CGCTTCAGCAGGCGCGGGCGGTTGGTCTCCAGCGCGCCGCCGCGTCCCCGCA
GGCCGTCCCACAGGCCCCAGCCCAGGCAGCGCAGCTTGAGCAGCTTGTCGCG
TTCGAGCAGGAGCACCGCGAGGCCCTGGGTCAGGGTCGGCAGGTTCGCCAGC
AGGGCCAGCGGCGAGGACCGGGCGTAGCGGCGCAGGACCAGCAGGCCGTTG
CGCGCCAGGTAGTAGCGGCGCAGCGGGGCGTGGTTCATCGCGCTGAGGCTGA
GACCGCCGAGGCGGCGGGTCTTGCGCGTGCCGATGCGGTGCTCGAGGACCAG
CCGCGGGTCGACGTACAGGGGCACGTCCAGCGCCTGGGCGCGCAGGCTGTAT
TCGGTGTCCACGTGGTCGATGAACAGTTCCTCGTCGAAGTGGCCGAGGCGCT
GGTAGGCCTCGCGGGTCAGCAGGCAGCCGGAGGAGATCAGGAACGAGGTGC
GCTGCGGGGTCGTCAGGCCGTCCAGAGACAATTGCCTGAGCGTCAGTCCGTC
GAGATGGATGGCCGGCAGGAAGCGCCGGTCACCCCGGTCGAAGATCCGTGGG
CCGAGCAGGCAGGCCTGACCGTTGCGCGCCTGCAGGTTGCGCCACTGGGCGG
CGAGGAAGGCGCCGCCGGGACGGGAGTCCTGGTCGAGCAGCAGCACACCCT
GCACGCCACGCCGGAATAGCGCGTCGAGTCCCTGGTTGAAGGCGCCGGCGAT
GCCCTGCCGGTTGCCGTGGTGCAGCACGGCGATGCCTTGCCCGCGCAGCCGG
GCATTGCGCTGCGGATCGCTGTGCGGTGAGTTGTCGACGGCAAGGAAGCGCA
GTTGCGGAAACGCCGCCGCCAGTTCGCCAAGGTGTTCCAGGTCGTCGTCGCC
AGGATTGAACAGTACCACCAGCACGCCCATGTCTATCCGGTCCAT

2. Complete sequence of rhamnosyltransferase (*rhl*C) from *Burkholderia thailandensis* E264, product length of 985 bp.

ATGACGATCCTGGGGGCGCTGGTGATTCTGTACGACCCGACGGACGAGCAGT
TGTCGGGGCTGGAGGCGCTCGCGCGCGACAGCGACGCGCTCGTGGTCGTGGA
CAACACGCCGCACGAGCACGCGGCGGCGCGCGAGCGGGTGCGTGCGCTGTCG
GCGCGGACGAACACGGTGTGGCGACACCACGGCAACCGGGGCGGGGTCGCG

GGCGGGTACAACGCGGGGCTGTCGGTGCTGTTCGCGCAGGGCGTCGAGGCGG
TCGCGCTGTTCGACCAGGACTCGACGGTGCCGGCCGGGTACTTCGAGCGGAT
GCGCGAGGCGTGCGCGCAACTGGGTGAGCAACCGGGCGCGCACGCGGGCGC
GTTCATCGCGGGCCCGCGGATCTACGACGCGAACGAGCAGCGCTTCCTGCCG
GAGCTGATGACGAGCGGGGTGACGGTGCGCCGCGTGCGGGTGGAGGGCGAG
ACGGCGCCGCAGCGCTGCGCGTTCCTGATCTCGTCGGGCAGCGTGATTTCGCG
GGCCGCGTACGCGCGGCTCGGTCGATTCGACGAGGCGCTGTTCATCGATCAC
GTCGACACCGAGTATTGCCTGCGCGCGCTCGCGCACAACGTGCCGCTGTACGT
GGTGCCGCCGCTCGTGCTGACGCACCGGATCGGCGCGCGGCGCCGGCACAAG
GTGGGGCCGTTCGAGCTGACGGCGATGCATCACGGGTGGTTGCGCCGATACT
ACGGCGCGCGCAACGCGATGCAACTGGGGCTGCAGTACGGCTTGCGGTTTCC
GGTGGCGCTGGTGCCGAATCTGCTGACGATATGGCAGGTGATCCAGGTGGTG
CTGTGCGAGCGGGAGAAGGGCGCGAAGCTGCGCGGGATCGCGCTGGGCGTG
CTCGACGGCCTGTTCGGGCGGCTGGGATCGTTCGACGATGCGCGCGCGGGCG
CGGCGGCGCGCGAGCCGGTGCGGCAGGAATGA

3. Complete sequence of the genes PA1131 and rhamnosyltransferase II (*rhl*C) from *Pseudomonas aeruginosa* #112, product length of 2370 bp.

CTAGGCCTTGGCCTTGCCGGAAGCTACGGACGCCACGGCCGGGCCGGCGAGG
CGCTTCAGCAGGCGCGGGCGGTTGGTCTCCAGCGCGCCGCCGCGTCCCCGCA
GGCCGTCCCACAGGCCCCAGCCCAGGCAGCGCAGCTTGAGCAGCTTGTCGCG
TTCGAGCAGGAGCACCGCGAGGCCCTGGGTCAGGGTCGGCAGGTTCGCCAGC
AGGGCCAGCGGCGAGGACCGGGCGTAGCGGCGCAGGACCAGCAGGCCGTTG
CGCGCCAGGTAGTAGCGGCGCAGCGGGGCGTGGTTCATCGCGCTGAGGCTGA
GACCGCCGAGGCGGCGGGTCTTGCGCGTGCCGATGCGGTGCTCGAGGACCAG
CCGCGGGTCGACGTACAGGGGCACGTCCAGCGCCTGGGCGCGCAGGCTGTAT
TCGGTGTCCACGTGGTCGATGAACAGTTCCTCGTCGAAGTGGCCGAGGCGCT
GGTAGGCCTCGCGGGTCAGCAGGCAGCCGGAGGAGATCAGGAACGAGGTGC
GCTGCGGGGTCGTCAGGCCGTCCAGAGACAATTGCCTGAGCGTCAGTCCGTC
GAGATGGATGGCCGGCAGGAAGCGCCGGTCACCCCGGTCGAAGATCCGTGGG
CCGAGCAGGCAGGCCTGACCGTTGCGCGCCTGCAGGTTGCGCCACTGGGCGG
CGAGGAAGGCGCCGCCGGGACGGGAGTCCTGGTCGAGCAGCAGCACACCCT
GCACGCCACGCCGGAATAGCGCGTCGAGTCCCTGGTTGAAGGCGCCGGCGAT

GCCCTGCCGGTTGCCGTGGTGCAGCACGGCGATGCCTTGCCCGCGCAGCCGG
GCATTGCGCTGCGGATCGCTGTGCGGTGAGTTGTCGACGGCAAGGAAGCGCA
GTTGCGGAAACGCCGCCGCCAGTTCGCCAAGGTGTTCCAGGTCGTCGTCGCC
AGGATTGAACAGTACCACCAGCACGCCCATGTCTATCCGGTCCATGATCGTTC
TTCTCCCGTAGGTCGAAGTTGCCAGGCCAGGACCAGCCCGGCCAGAACGAGA
AGGGCGCCGGCGAGGAATGGCGCGCCGGCCAGGGGCAGCGGCGCGAGCGGA
CCGCTGCCCCAGTGGAACAGGCCGCTCATCAGCGGCGGACCGACGATCGCGG
CGAGGCTCATCAGGCTGCTCAGCACGCCCTGCAACTCGCCCTGGCGGTCGAC
CGGCACGCGGGCCGAGAGCAGCCCCTGCATGGCCGGGGTGGCGAGGCTGCCG
AGCGCGAAGGGCAGCAGCGCGCAGACCAGCCAGAATGACGAGTCGACCAGG
GCGAACAGCAGCAGGCCGCAGCCTTGCAGGGCGAGGCCCAGGCGCAGCAGG
CGGGCGTCGTCCAGGCGCCGCTTGCAGAGGTTCACGCCGAGGGTCTGGGCGA
GCACCGCGAGCACGCCGTAGAGGGCCAGCGAGTAGCCGATCCAGGCGCTGCT
CCAGTGAAACTTCTCGATCACGAAGAACGGCCAGACCACCATCACCGCCTGC
AAGCCGAGGAATACCAGGGCAAGCACCGCCAGCAGGCGTCCGACCCCCGGTT
GCCGAGCCAGGCCGCTGATCGAGCGCAAGGCATTCATCCGCCTCGGGTCCAG
GCGGCGGCGTCGCGTCGGGGGCAGGGTTTCCTCGAGGAACAGGCCGGCGAGC
AGGGCGTTGAGCAGGCACAGGCCGGCGGCCAGCAACAGCGGCAGCGTCGTG
CCGTGCACCGCCAGCAGCCCACCGAGGGCGGGGCCGAGGATCATGCCCAGGG
CGAGGCCGGCGTACAGCCAGCCGAAGTGCCGGGTGCGCTGCCCGTGCGTGCC
GAGGTCAGCCGCGCAGGCCATCGCGGTGGCCACGCTGGCGCCGGTGAGCCCG
GCCAGCGCGCGACCGAGGAACAGCATCCAGAGGCTGTCGGCCAGCGCCAGC
AGCAGATAGCTGAGGGCGAAGCCGAGCATCGCCAGGACCAGGACGGGGCGG
CGTCCGAAGCGGTCGCTGAGGCTGCCGAGGACCGGCGAAAAGAACAATTGCA
GCAGCGCGAAGGTCATCACCAGGGCGGCGCCCCAGGTGGCCGCGTCGCGGAC
CGCCAGCGGCGCCACGCTGCCGATCAGCGTCGGCAGCAGGGGCACGATCAGG
CCGACGCCAGCGGCATCCAGCAGGCAGGTGAGGAACAGCAGAGGCAGGACG
CGTTTCGCGCCGGGACCGTGTTCCCGCGTGGCGGAGGGGCAGAGGCTGGTCG
TGGACACGCCAGGATCCTCCCGGCGAACACAGGAAATCCTGATCTTGGGACG
CCAGCGAGGGCAAGGGAAACTACCGGAATTCACAGGTTCGACGAACGGTCGC
GGCAACGCCGGCGGGCGGCGTGATGTCATA

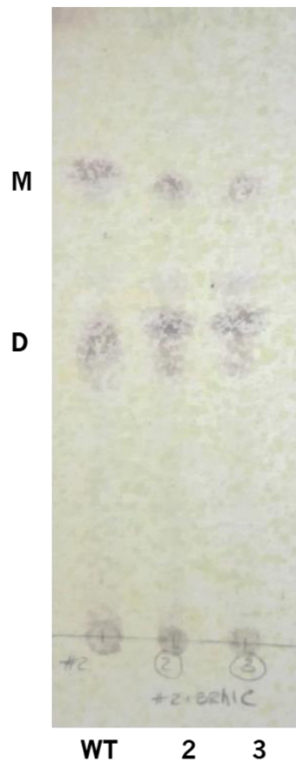

Figure 6.S1. TLC of the mono- (**M)** and di-rhamnolipids (**D**) profile at the end of the fermentation at 37ºC at 180 rpm by *P. aeruginosa* #2 (**WT**) and two transformants of *P. aeruginosa* with pVLT31_BRhlC with 0.4 mM of IPTG (**2** and **3**).

CHAPTER 7

GENERAL CONCLUSIONS AND FUTURE PRESPECTIVES

"Life is the art of drawing sufficient conclusions for insufficient premises"

Samuel Butler

In this chapter the main conclusions from the present thesis are compiled. More detailed conclusions can be found at the end of each individual chapter. In addition, recommendations for further research in this field are discussed.

7.1. GENERAL CONCLUSIONS

The main purpose of this thesis was to evaluate the antifungal and anti-mycotoxin activity from the rhamnolipids (mixture and congeners) produced by *Pseudomonas aeruginosa* against different *Aspergillus* species and engineering *P. aeruginosa* strain through the production of the best and effective rhamnolipids. For this purpose, the culture medium was optimized using different agro-industrial residues as carbon and nitrogen sources, towards the process simplification and cost reduction, which are attractive from the industrial point of view.

The main conclusions drawn from this thesis are summarized below:

- The screening of different agro-industrial residues revealed that the culture medium using a combination of the three agro-residues (corn steep liquor (CSL), molasses and olive oil mill waste water (OMW) at optimal concentrations) without any previous treatment and without additional supplements, was the most attractive medium for the rhamnolipids production by *P. aeruginosa*, presenting the highest yields, good emulsifying indexes and lowest critical micelle concentrations (*cmc*).

- The high amount of long-chain fatty acids in the composition of OMW make this residue a great inducer of rhamnolipids production by *P. aeruginosa*. OMW is a hazardous waste and their potential use as substrate in bioprocesses has a positive effect reducing their environmental impact and also the cost of the culture medium.

- Comparing assays performed in flasks and 2L reactor using medium CSL and molasses (M) without OMW, it was found that the amount of rhamnolipid produced in reactor was lower although exhibiting a lower *cmc*, thus meaning a higher efficiency. On the contrary, when using the medium CSLM supplemented with 25% of OMW, the amount of rhamnolipids produced in the reactor was higher and exhibited similar *cmc* values. The air injection in the top of the reactor revealed to be a good strategy to avoid the foam associated to biosurfactants production. This approach could simplify and reduce the costs associated with the biosurfactants production by avoiding the use of antifoam agents and complex reactor design and construction, particularly in an industrial setup.

- The ratio of mono- and di-rhamnolipids produced by *P. aeruginosa* depends on the culture medium used. The medium CSLM supplemented with OMW led to a higher proportion of mono-rhamnolipids comparing with the medium CSLM without OMW. The selection of the culture medium that leads to an increase of a specific congener could be advantageous

- The cell-free supernatant, obtained at the end of the fermentation by *P. aeruginosa*, exhibited a high antifungal activity against *Aspergillus niger* MUM 92.13 and *Aspergillus carbonarius* MUM 05.18. Moreover, it was demonstrated that the di-rhamnolipid congener was the responsible for the antifungal activity found for the crude rhamnolipids mixture. A direct relationship between the antifungal activity of the rhamnolipids and the aggregation behavior was demonstrated. The structures formed by the rhamnolipids increased with increasing of NaCl concentrations, being the effect more pronounced in the case of di-rhamnolipids.

- The rhamnolipids produced by *P. aeruginosa* #112 and *Burkholderia thailandensis* E264 inhibited the aflatoxins production from *Aspergillus flavus* MUM 17.14, being the rhamnolipids produced by *P. aeruginosa* at lower concentrations the more efficient. The inhibition of aflatoxins production by *A. flavus* MUM 17.14 by the rhamnolipids could be explained by the strong inhibition expression of the genes *afl*C and *afl*Q involved in the aflatoxins biosynthesis. The several damages observed in the internal hyphae content could also justify the reduction of the aflatoxins production found.

- The engineering of *P. aeruginosa* in order to increase the production of di- was accomplished with the recombinant plasmid pVLT31_PsRhlC. This transformant showed similar yields comparing with the wild type and a reduction of approximately 60% of the mono-rhamnolipids produced.

7.2. FUTURE PRESCPECTIVES

Several questions aroused from the findings of this thesis that warrant further research. Future directions on rhamnolipids production and their potential application as a biological agent are summarized below:

- The use of the different agro-residues as a culture medium for the rhamnolipids production gave very interesting perspectives towards the economic feasibility of their industrial production process. According to the culture medium used, *P. aeruginosa* produces different ratio of mono- and di-rhamnolipids, thus it would be advantageous to evaluate the potential of new agro-residues combinations to increase the production of the specific congener at industrial scales.

- Despite the promising results obtained using the 2L reactor by reducing the foam formed with air injection on top of the culture medium, it is still a challenge to produce rhamnolipids at an industrial scale. For that reason, a bioreactor with a suitable design is required in order to promote a good agitation and oxygen availability for the optimal microorganism growth and foam reduction.

- Rhamnolipids can play an important role as antifungal and anti-mycotoxigenic agent for agriculture applications being a promising alternative to the chemical fungicides commonly used. Furthermore, in the future these compounds, both as mixtures and specific congeners, should be tested against other contaminant fungi and also, against fungi consortia. Moreover, additional studies are required to unravel the mechanisms underlying the effect of rhamnolipids in the aflatoxin biosynthesis that could be helpful for the development of more effective biological agents.

- Since the presence of NaCl changes the rhamnolipids structure and consequently increase their antifungal activity, it would be interesting to evaluate different formulations (e.g. combining them with other divalent cations, biosurfactants, optimizing pH) that could be useful for the development of more effective antifungal agents with better and improved properties. Given the direct relationship between the rhamnolipids antifungal activity and their aggregation behavior, it will be interesting to evaluate if this relationship would improve their other biological activities (e.g. antimicrobial activity, anti-tumor, among others).

- Regardless of the increase of di-rhamnolipids production by the transformant with the recombinant plasmid pVLT31_PsRhlC, the yields obtained are still not economically attractive and, for that reason, additional studies need to be performed. For example, it would be interesting to evaluate the rhamnolipids production and the production profile by the transformants using the culture medium with agro-residues and also to study their biological activity.